



# Exploring Dengue Virus Entry through Small Molecule Inhibition and Mutagenesis of the Envelope Protein

## Citation

Clark, Margaret Jean. 2012. Exploring Dengue Virus Entry through Small Molecule Inhibition and Mutagenesis of the Envelope Protein. Doctoral dissertation, Harvard University.

## Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:10416139>

## Terms of Use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA>

## Share Your Story

The Harvard community has made this article openly available.  
Please share how this access benefits you. [Submit a story](#).

[Accessibility](#)

© 2012 – Margaret Jean Clark

All rights reserved

## **Exploring Dengue Virus Entry Through Small Molecule Inhibition and Mutagenesis of the Envelope Protein**

### **Abstract**

Over one-third of the world's population is at risk for infection with dengue virus (DENV), a mosquito-borne virus that can cause a severe febrile disease. There are no specific treatments available for dengue infection, and much remains unknown about how DENV interaction with the host cell leads to a successful infection. This dissertation examines DENV entry using small molecule inhibitors and mutagenesis of the envelope (E) protein, the major protein on the viral surface.

This work grew from our initial observation that small molecule GNF-2 is capable of lowering DENV yield when present at two separate points during DENV infection. Treatment of infected cells with GNF-2 post-entry significantly lowered DENV yield, most likely due to GNF-2's documented activity against Abl kinase. However, we also observed that treatment of virus inocula with GNF-2 prior to cellular infection significantly lowered DENV yield. We discovered that GNF-2 bound directly to the dengue virion and co-localized with DENV envelope protein shortly after cellular infection. Using GNF-2 as a scaffold, we performed a structure-activity relationship study and identified twenty-one compounds that have similar or increased potency as GNF-2 when pre-incubated with virus. Using a subset of compounds from this study, we demonstrated that they block completion of DENV fusion *in vitro*, suggesting that the

compounds inhibit DENV entry by preventing the completion of viral fusion inside cellular endosomes.

In experiments complementing the mechanism of action studies, we selected for inhibitor-resistant virus by passaging virus in the presence of small molecules. We identified a single point mutation in the envelope protein located in the domain I/II interface that enhanced viral entry and conferred resistance to virus particles against select compounds in a single-cycle reporter virus system. Further examination of this E protein “hinge region” found that mutations in this area may affect both release and entry of reporter virus particles. The work presented in this dissertation may inform the design of future small molecule inhibitors of DENV as well as increase our understanding of how point mutations in the DENV E protein can influence viral entry and other steps of the viral life cycle.

## Table of Contents

<b>Abstract</b>	iii
<b>Table of Contents</b>	v
<b>List of Figures</b>	vi
<b>List of Tables</b>	ix
<b>Acknowledgments</b>	x
<b>Chapter 1:</b> Introduction	1
<b>Chapter 2:</b> Characterization of GNF-2, a small molecule inhibitor of cellular Abl kinases, as an inhibitor of dengue virus entry	23
<b>Chapter 3:</b> Exploring the mechanism of action of select GNF-2 analogs during dengue virus entry	64
<b>Chapter 4:</b> Exploring potential resistance mutations in DENV structural proteins against small molecule entry inhibitors	99
<b>Chapter 5:</b> Discussion	139
<b>Appendix A:</b> Table of GNF-2 analogs active during DENV entry	152

## List of Figures

<b><u>Figure</u></b>	<b><u>Title</u></b>	<b><u>Page</u></b>
<b>1-1</b>	<b>Organization of the dengue virion</b>	<b>6</b>
<b>1-2</b>	<b>Crystal structures of the DENV2 envelope protein</b>	<b>8</b>
<b>1-3</b>	<b>Schematic of DENV fusion</b>	<b>11</b>
<b>2-1</b>	<b>Structures of GNF-2 and imatinib</b>	<b>28</b>
<b>2-2</b>	<b>GNF-2 lowers DENV titer when pre-incubated with virus inoculum</b>	<b>31</b>
<b>2-3</b>	<b>Structure of NITD6</b>	<b>32</b>
<b>2-4</b>	<b>GNF-2 has additive effects on DENV titer</b>	<b>34</b>
<b>2-5</b>	<b>c-GNF-2 structure and dose-response titration against DENV2</b>	<b>36</b>
<b>2-6</b>	<b>GNF-2-CY5 structure and dose-response titration against DENV2</b>	<b>38</b>
<b>2-7</b>	<b>GNF-2-CY5 co-localizes with DENV E protein</b>	<b>39</b>
<b>2-8</b>	<b>GNF-2-CY5 is not taken up in presence of VSV</b>	<b>40</b>
<b>2-9</b>	<b>GNF-2-biotin structure and dose-response titration against DENV2</b>	<b>42</b>
<b>2-10</b>	<b>GNF-2-biotin interacts with purified DENV2</b>	<b>43</b>
<b>2-11</b>	<b>GNF-2-FITC interacts with recombinant soluble DENV2 E protein</b>	<b>45</b>
<b>2-12</b>	<b>Dose-response titration of GNF-2 and c-GNF-2 against DENV1, 3, and 4 strains</b>	<b>47</b>

<b><u>Figure</u></b>	<b><u>Title</u></b>	<b><u>Page</u></b>
<b>3-1</b>	<b>Structures of small molecules 2-12-2 and 8-24-3</b>	<b>70</b>
<b>3-2</b>	<b>Small molecules lower DENV titer when pre-incubated with virus</b>	<b>71</b>
<b>3-3</b>	<b>DENV titer is reduced when free compound is removed prior to infection</b>	<b>73</b>
<b>3-4</b>	<b>Inhibition by compounds is lost with increasing initial MOI</b>	<b>75</b>
<b>3-5</b>	<b>Pre-incubation at 4°C causes loss of inhibition by compounds</b>	<b>76</b>
<b>3-6</b>	<b>Pre-incubation time with DENV2 affects magnitude of inhibition</b>	<b>78</b>
<b>3-7</b>	<b>Inhibition by compounds may be reversible</b>	<b>79</b>
<b>3-8</b>	<b>Compounds do not block DENV2 attachment to cells</b>	<b>82</b>
<b>3-9</b>	<b>Compounds do not inhibit DENV association with target membranes</b>	<b>84</b>
<b>3-10</b>	<b>Compounds prevent completion of viral fusion with target membranes</b>	<b>85</b>
<b>4-1</b>	<b>Crystal structure of the DENV2 envelope protein</b>	<b>103</b>
<b>4-2</b>	<b>Passaging of DENV2 in the presence of selected compounds</b>	<b>107</b>
<b>4-3</b>	<b>Structure of compound 7-148-6</b>	<b>108</b>

<b><u>Figure</u></b>	<b><u>Title</u></b>	<b><u>Page</u></b>
<b>4-4</b>	<b>Compound 7-148-6 inhibits DENV titer when pre-incubated with virus inoculum</b>	<b>109</b>
<b>4-5</b>	<b>Compound 7-148-6 does not affect virus attachment to cells and prevents completion of DENV fusion</b>	<b>110</b>
<b>4-6</b>	<b>Location of residue 196 on DENV2 E protein</b>	<b>112</b>
<b>4-7</b>	<b>DENV structural protein mutations affect virus entry in a single-cycle infection assay</b>	<b>115</b>
<b>4-8</b>	<b>RVPs with DENV structural protein changes show decreased sensitivity to compound 7-148-6</b>	<b>117</b>
<b>4-9</b>	<b>Location of selected residues for mutagenesis on the DENV2 envelope crystal structure</b>	<b>119</b>
<b>4-10</b>	<b>Changes in the DENV E protein DI/DII interface affect production and entry of RVPs</b>	<b>121</b>
<b>4-11</b>	<b>DENV envelope protein mutations affect virus particle production and entry in a single-cycle infection assay</b>	<b>123</b>
<b>4-12</b>	<b>E(Q52A) and E(Q131A) may confer resistance to small molecule entry inhibitors</b>	<b>124</b>



## **List of Tables**

<b><u>Table</u></b>	<b><u>Title</u></b>	<b><u>Page</u></b>
<b>1-1</b>	<b>Previously published DENV entry inhibitors</b>	<b>14</b>
<b>2-1</b>	<b>Small molecule protein kinase inhibitors that inhibit DENV infection</b>	<b>27</b>

## **Acknowledgements**

There are many people to whom I owe a debt of gratitude; I would not have made it through my time in graduate school without both personal and professional support from many corners. First and foremost, I want to thank my family. They are the most amazing group of people, and my favorite times are when we are all gathered. My parents always listened, supported, and encouraged me in every endeavor. My sisters, Katy and Elizabeth, are amazing, strong women, and I am lucky to be related to them. Finally, my devoted husband Bruno, who has been eternally patient at my ever-changing timeline, supported me in every way possible despite the physical distance, and allowed me to finally understand selfless love and commitment.

My second thanks goes to my advisor, Dr. Priscilla L. Yang. She has been invested in me as a scientist and as an individual from the moment I began my rotation, and I cannot thank her enough on either level. With her guidance and support, I have grown more than I thought possible as a scientist and experimentalist. I am also in debt to other members of the Yang lab, past and present: Mary Rodgers, Valerie Villareal, Mike Vetter, Margot Carocci, Christie Hershey, and Melissanne de Wispelaere.

As a whole, the graduate student community in the Virology program is exceptional, and I have greatly enjoyed it in my time here. Thanks to several of my peers have greatly influenced or helped in my research (or in preserving my sanity, which is related): Jennifer Spangle, Anna Bruchez, Silvia Piccinotti, Melissa Laird, and Shaila Rahman. A special thanks to Aaron Schmidt, for his valuable support both personally and experimentally.

## **Chapter 1:** Introduction

## SIGNIFICANCE

There are over 50 million cases of dengue fever worldwide each year, and almost forty percent of the world's population is at risk for infection by the causative agent, dengue virus (DENV) (WHO, 2012). DENV is transmitted by the bite of infected mosquitoes, predominantly *Aedes aegypti*, and there are currently no specific treatments or therapies available. Despite its global presence, much remains unknown about basic biological processes of the virus and its life cycle, as historically most research has focused on patient care. One possible avenue to blocking dengue virus infection may be to inhibit viral entry, as has been done for HIV using T-20, a peptide that binds and inhibits viral glycoprotein-mediated membrane fusion (Kilby et al., 1998).

In broader efforts to use chemical biology to interrogate cellular factors and processes required for successful DENV infection, we conducted a screen of known kinase inhibitors and identified several small molecules that had potent activity against DENV in cell culture (Chu and Yang, 2007). Subsequently, while exploring the function of GNF-2, a small molecule of intracellular Abl kinases, we found that GNF-2 inhibited DENV infectivity when incubated with virus inoculum prior to cellular infection. The work presented in this study explores the activity of GNF-2 and its derivatives as DENV entry inhibitors. Small molecule inhibitors have traditionally been useful as molecular probes in studying the function of their respective targets. Here, we have approached the mechanistic study of GNF-2 and related compounds blocking viral entry as a means by which to discover more about the DENV entry process and the structure and function of the DENV envelope protein, which is responsible for mediating all steps of viral entry. This knowledge may inform the development of future antivirals.

## **INTRODUCTION**

### **Dengue virus as an emerging global human pathogen**

In the past fifty years, dengue virus (DENV) has become a major global human pathogen; the World Health Organization (WHO) estimates that there are currently between 50 and 100 million dengue fever cases worldwide each year, and over 2.5 billion people are at risk for contracting the disease. Cases of dengue fever have increased alarmingly over the past forty to fifty years in both frequency and geographic spread. Prior to 1970, only nine countries had reported dengue outbreaks, while by 2010 over 100 countries were considered to have endemic dengue (WHO, 2012). While the recent increase may be due to increased reporting and surveillance, evidence suggests that the number of dengue cases worldwide is actually under-reported (Amarasinghe et al., 2011; WHO, 2012). The explosion of DENV cases has been attributed to increased urbanization and the accompanying spread of the primary carrier of DENV, the mosquito species *Aedes aegypti*. Mosquitoes are infected with DENV when they bite an infected human with a high viral load, and, after a short incubation period during which the virus works its way to the salivary glands, the insects transmit the virus for the rest of their lives (Gubler, 1976). It has also been shown that DENV can be transmitted vertically from mosquitoes to their progeny (Rosen, 1987).

### **Dengue pathogenesis**

Dengue fever is the most common form of disease caused by DENV. It is characterized by a high fever, joint pain, rash, and flu-like symptoms that can last from two to seven days and typically appear four to seven days after contracting the virus

(Lindenbach and Rice, 2001). Although rarely fatal, particularly if patients are given supplemental fluids, it can be a painful and debilitating disease, so much that it is commonly referred to as “breakbone fever.” A more serious presentation of dengue virus infection is severe dengue, a form of the disease in which a patient can experience bleeding, fluid buildup, and plasma leakage. This form of dengue, which has a 20-30% mortality rate if left untreated, is the leading cause of hospitalization and death among children in many endemic areas (WHO, 2012).

There are four serotypes of DENV (DENV1, DENV2, DENV3, and DENV4) that are defined by their antigenic differences (Calisher et al., 1989). It is not clear if certain DENV serotypes cause more severe illness (Balmaseda et al., 2006; Vaughn et al., 2000), but it is known that surviving a specific serotype provides life-long immunity to that serotype while providing only short-term protection against other serotypes (Lindenbach and Rice, 2001). In fact, surviving one serotype increases the risk of developing severe dengue if a person subsequently is infected with a different serotype (Alvarez et al., 2006). This increased risk of severe disease is likely due to a phenomenon known as antibody-dependent enhancement (ADE), in which antibodies developed against one serotype bind to but do not neutralize virions of a different DENV serotype. These antibody-coated virus particles experience enhanced binding and infection of cells expressing Fc receptors, such as macrophages, which then leads to higher viremia in patients and more severe manifestations of the disease (Peiris and Porterfield, 1979). ADE is of increasing concern to the public health community as DENV spreads around the globe; as of 2004, many regions of the world reported all four serotypes circulating in the population (Mackenzie et al., 2004). Due to the risk of ADE, vaccine development for

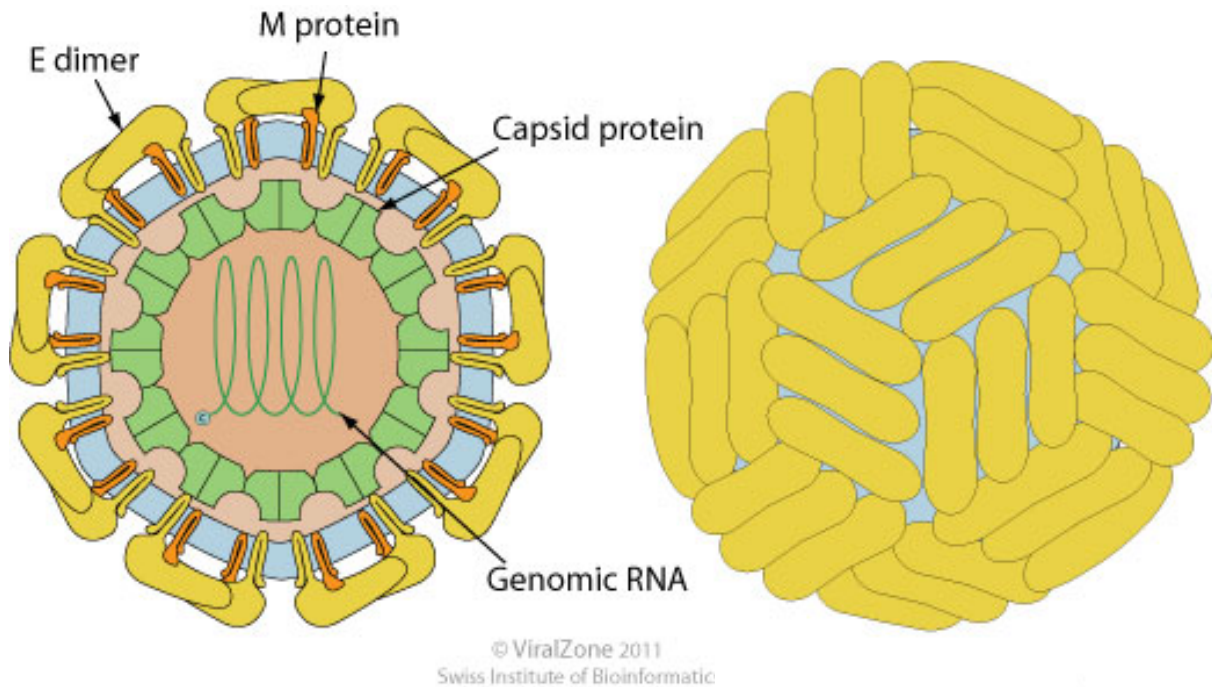
DENV is a major challenge, as vaccines must elicit a protective antibody response against all four serotypes.

Despite the rapid global spread of dengue virus and large risk of infection to the world's population, there is a dearth of knowledge about many basic DENV processes. Historically, most research has focused on patient health, symptoms, outbreaks, genotyping, or epidemiology. Thus, while the need for specific treatments and therapeutics is urgent as the virus continues to spread to new areas of the world, the design of such antivirals is severely hampered by lack of knowledge about how DENV successfully carries out infection and replication.

### **Dengue virus life cycle**

Dengue virus is a member of the *Flaviviridae* family, which includes other human pathogens such as yellow fever virus (YFV), Hepatitis C virus (HCV), and the closely related West Nile virus (WNV). Dengue virions contain a positive-sense, single-stranded RNA genome, approximately 11kb in length, that associates with the small viral capsid (C) protein (Lindenbach and Rice, 2001). The genome contains short non-coding regions at both the 3' and 5' end that have been shown to be important for both translation and replication (Holden et al., 2006; Kinney et al., 2005). The genome is translated as one polyprotein that is co- and post-translationally cleaved into three structural (C, prM, and E) and seven nonstructural proteins (NS1, NS2A/B, NS3, NS4A/B, and NS5).

DENV has a lipid envelope and mature virions are approximately 50 nm in diameter. There are 180 copies of the envelope (E) protein on the virion surface that form 90 homodimers arranged in a tight herringbone structure [Figure 1-1, (Kuhn et al., 2002)].



**Figure 1-1. Organization of the dengue virion.** Schematic of the mature dengue virion.

(*Left*) Cutaway showing organization of structural proteins on the exterior of the virion

with the genomic RNA inside. (*Right*) Herringbone arrangement of E protein

homodimers on the virion surface. Used with permission from ViralZone

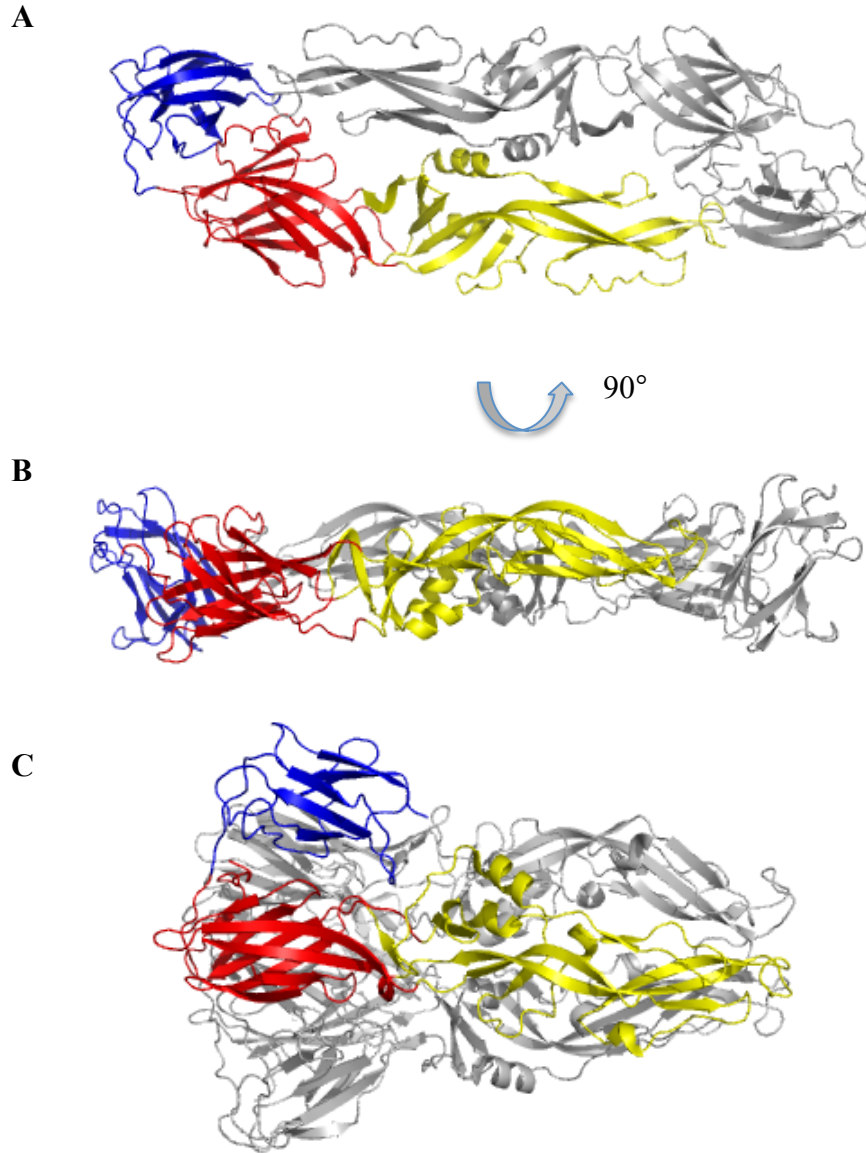
([www.expasy.org/viralzone](http://www.expasy.org/viralzone), Swiss Institute of Bioinformatics).



These E protein dimers mediate binding to cells, after which the virus is taken up by clathrin-mediated endocytosis into endosomes (Acosta et al., 2008; van der Schaar et al., 2008), where the low pH triggers viral fusion (Gollins and Porterfield, 1986). The viral genome is released into the cytoplasm, where it undergoes translation and replication in membrane-associated complexes in the perinuclear region (Westaway et al., 1997). The polyprotein is cleaved by viral and cellular proteases and nascent virions are assembled by budding into the endoplasmic reticulum (Mackenzie and Westaway, 2001). New virions are exocytosed through the cellular secretory pathway, during which prM, a chaperone protein that protects premature triggering of the E protein, is cleaved by furin (Stadler et al., 1997) and the E protein rearranges into homodimers (Stiasny et al., 1996).

### **DENV envelope protein**

The DENV envelope (E) protein is the major glycoprotein present on the exterior of the mature virion and mediates all aspects of viral entry. E consists of three domains, a stem region, and a transmembrane domain. The pre-fusion and post-fusion crystal structures of DENV2 soluble envelope protein, containing domains I-III, have been solved and are similar in appearance to other flavivirus E proteins [Figure 1-2; (Modis et al., 2003, 2004)]. Domain III contains an immunoglobulin-like region that is believed to be the receptor-binding domain, while finger-like domain II contains the fusion loop at its distal end (Mukhopadhyay et al., 2005). The stem region, although not solved as part of the crystal structures, is thought to consist of two highly-conserved amphipathic helices and a loop that lie close to the viral membrane in the pre-fusion conformation and play an



**Figure 1-2. Crystal structures of the DENV2 envelope (E) protein.** (A) Pre-fusion E protein homodimer as viewed from above virion surface. Domains I, II, and III of a single monomer are shown in red, yellow, and blue, respectively. Stem region of E is not shown. (B) Side view of the E homodimer. The virion membrane would be located underneath the structure in this orientation. (C) Post-fusion E trimer. Note the large movement of domain III in a monomer relative to the pre-fusion dimer. PDB IDs: 1OAN [dimer; (Modis et al., 2003)], 1OK8 [trimer; (Modis et al., 2004)].

important role in creation and stabilization of the post-fusion E trimer (Zhang et al., 2003a).

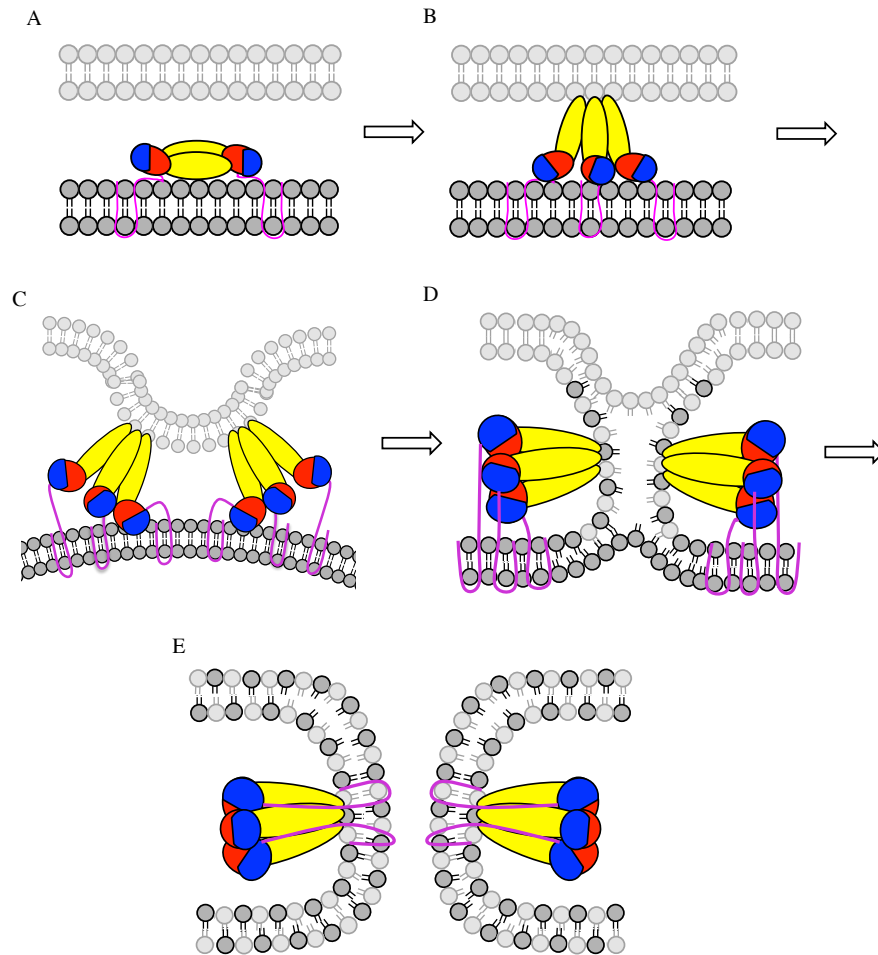
Throughout the viral life cycle, the E protein undergoes a series of conformational rearrangements and protein associations. Initially expressed as part of the polyprotein, E is translocated into the ER lumen and quickly forms a heterodimer with prM, which acts as a chaperone protein and is necessary for proper folding of E (Konishi and Mason, 1993). These E/prM heterodimers appear as trimer spikes on the surface of newly assembled, immature virions (Zhang et al., 2003b). As the virus passes through the low pH of the trans-Golgi network, the E protein rearranges into dimers and the pr peptide is cleaved by the cellular protease furin but remains associated (Yu et al., 2008), protecting the fusion loop to prevent premature triggering of the post-fusion trimer rearrangement (Zhang et al., 2004). The remnant of prM, M, is a small (~8kD) protein that sits underneath the E homodimer on the virion surface and consists of an alpha helix that remain membrane associated (Zhang et al., 2003a). As the dengue virion is exocytosed from the cell, the pr peptide is released, and the E protein is found in its pre-fusion, homodimer, herringbone arrangement on the virion surface (Figure 1-1) (Zhang et al., 2003a).

Mature virions bind to cells via interactions with unknown receptor(s), although several classes of surface proteins, including DC-SIGN (Tassaneetrithep et al., 2003) and glycosaminoglycans (Chen et al., 1997), have been suggested to play supporting roles. Once bound to cells, the virus is taken up into clathrin-coated pits and transported to endosomal compartments where viral fusion occurs (van der Schaar et al., 2008). The exact trigger for DENV fusion remains unclear, as a study using flavivirus tick-borne

encephalitis virus (TBEV) showed that protonation of a key histidine residue was responsible for triggering fusion (Fritz et al., 2008), while another study in WNV showed that no single histidine residue on E was necessary for fusion initiation (Nelson et al., 2009). The optimal pH for triggering DENV fusion is also unknown; WNV fusion has been reported at pH levels as high as 6.9 (Moesker et al., 2010), although the threshold for DENV is typically agreed to be around 6.3 (Lee et al., 1997; Nelson et al., 2009).

### **DENV fusion**

The DENV E protein is a class II viral fusion protein. The crystal structures of the pre-fusion dimer and post-fusion trimer have been solved, revealing several large conformational changes (Figure 1-2) (Modis et al., 2003, 2004). Fusion is hypothesized to be a multi-step process and have several distinct structural intermediates (Figure 1-3). Upon first encountering low pH, the E homodimer is thought to dissociate into monomers, allowing exposure of the fusion loop and extension of domain II away from the viral membrane. The fusion loop, located at the tip of domain II distal from the viral membrane, inserts into the target membrane and domain III folds back, allowing for the two membranes to be brought in proximity. The alpha helices of the stem region of E begin to “zip up” along the E protein domains. As the stem region extends and increases its contact with the E protein, hemifusion occurs, in which the outer lipids of each membrane mix. This is followed by the final pore formation, which allows for the viral genome to be released into the cytoplasm. It is unclear how many E proteins are necessary to mediate successful fusion pore formation, and it is possible that the fusion pore may “flicker” before opening completely (Harrison, 2008). Supportive mechanistic



**Figure 1-3. Current model of DENV fusion.** (A) DENV envelope protein homodimer in its pre-fusion state, resting parallel to the virus membrane. Domains I, II, and III are shown in red, yellow, and blue, respectively. Stem region shown in pink. (B) Triggered by low pH, the E protein dimer dissociates and the fusion loop, located at the tip of domain II, inserts into target membrane. E protein rearranges into trimers. (C) Domain III folds back and stem regions begin to zip up the outside of the trimer, bringing membranes in proximity. (D) Hemifusion occurs, in which the outer lipid membranes of the virus and target membrane mix. (E) Stem region completes zipping up, fusion pore is formed, and content mixing occurs between virus and target membrane. *Adapted from (Harrison, 2005)*

evidence for the extended structural intermediate of Class II fusion proteins has been shown in alphaviruses (Sánchez-San Martín et al., 2008), while a study showing that stem region peptides interfere with late stages of fusion provide support for interactions of the stem region alpha helices with the post-fusion trimer (Schmidt et al., 2010).

Despite the obvious importance of the DENV E protein in entry, much remains unknown about how changes in E structure or sequence influence virus entry or other aspects of the viral life cycle. Two studies have suggested that changes in residues around the domain I/II interface may alter the optimal pH of viral fusion (Beasley and Aaskov, 2001; Lee et al., 1997), and this region has been suggested to act as a “hinge” during the movement of domain II that occurs during viral fusion (Modis et al., 2003). One recent study performed mutagenesis in this region and found that many amino acid changes altered either growth kinetics or fusion ability in both mammalian and insect cells (Butrapet et al., 2011). Recent work from our lab and others has shown that residues in the E DI/DIII linker region (de Wispelaere and Yang, 2012) or stem region (Hsieh et al., 2010; Lin et al., 2011) can impact virus assembly. These studies suggest that the E protein may play a role in steps of the viral life beyond mediating entry.

### **Inhibition of DENV entry**

Since the publication of T-20 as a peptide inhibitor of HIV entry (Kilby et al., 1998), viral entry has been considered a viable target for antivirals. For DENV, the first reported instances of inhibitors that acted at entry were peptides loosely derived from the stem region of E (Hrobowski et al., 2005). These peptides had an EC<sub>50</sub> value of 10-20

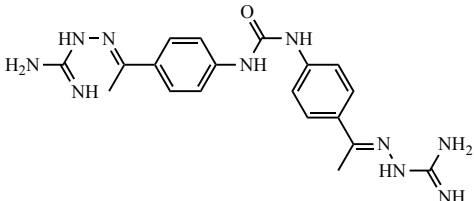
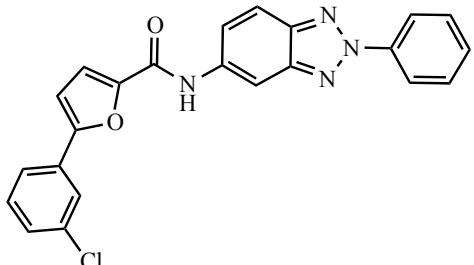
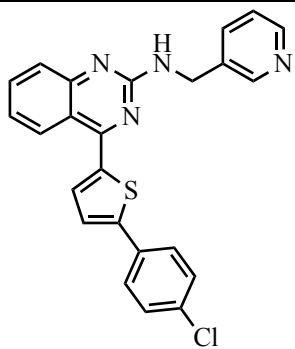
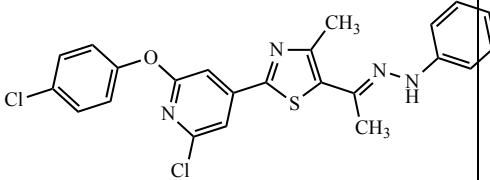
$\mu\text{M}$  when pre-incubated with virus inocula and present during viral infection, but no further exploration into the mechanism of action was explored.

More recent studies have focused on small molecule, rather than peptide, inhibitors of DENV entry (Table 1-1). Many of the studies used high-throughput computational screening techniques to identify molecules predicted to bind in the domain I/II interface. When the pre-fusion dimer of the DENV2 E protein was crystallized, a single molecule of beta-octo-glucoside (BOG), a detergent used in the crystallization buffer, was discovered tucked in this region. This area of the E protein was subsequently dubbed the “BOG pocket,” and it was hypothesized that a small molecule occupying this same space may prevent DENV fusion (Modis et al., 2003).

The first antiviral discovered based on high-throughput screening against the BOG pocket was published in 2008 (Zhou et al., 2008). The lead compound, P02, interacted with the recombinant dengue E protein as detected by NMR and also competed with BOG for binding. However, in cellular assays, the compound was tested against a yellow fever reporter virus, which, while a flavivirus, has only 44% envelope protein sequence similarity to DENV. In addition, P02 was found to be active against a YFV replicon that lacks E and other viral structural proteins, suggesting that the compound has additional targets downstream of entry that may be responsible for the anti-DENV activity observed in cell culture. In fact, the  $\text{IC}_{50}$  value against the virus ( $13\ \mu\text{M}$ ) was similar to the  $\text{IC}_{50}$  against the replicon ( $17\ \mu\text{M}$ ), complicating interpretation of P02’s antiviral mechanism(s) of action.

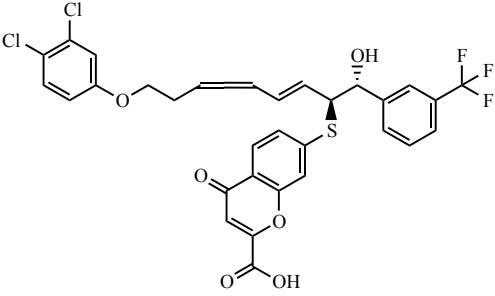
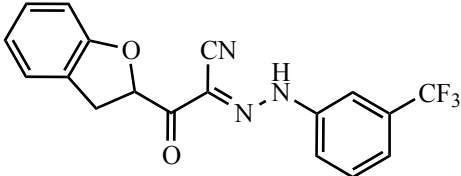
A later study explored other potential small molecule inhibitory sites on E and identified a compound hypothesized to make contacts with domains II and III that

**Table 1-1. Previously published inhibitors of DENV entry**

Inhibitor name <i>Source</i>	Structure	Activity (DENV2)	Test fusion of DENV?	Activity ag. other flaviviruses?
DN59 peptide <i>Hrobowski et al, 2005</i>	N/A	EC <sub>50</sub> 10 μM	No	WNV (EC <sub>50</sub> 10 μM)
P02 <i>Zhou et al, 2008</i>		Not tested	No	YFV (EC <sub>50</sub> 13 μM)
R1 <i>Yennamalli et al, 2009</i>		EC <sub>50</sub> 4 μM	No	Not tested
NITD6 <i>Wang et al, 2009</i>		EC <sub>50</sub> 119 nM	No	DENV1 (108 nM), DENV3 (496 nM), DENV4 (334 nM), YFV (470 nM), JEV (1.42 μM), WNV (564 nM)
A5 <i>Kampmann et al, 2009</i>		IC <sub>50</sub> 1.2 μM	Yes (Fusion from within)	Kunjin (3.8 μM), YFV (1.6 μM)



**Table 1-1, continued. Previously published inhibitors of DENV entry**

Inhibitor name <i>Source</i>	Structure	Activity (DENV2)	Test fusion of DENV?	Activity ag. other flaviviruses?
NITD448 <i>Poh et al, 2009</i>		IC <sub>50</sub> 9.8 μM	Yes <i>In vitro</i>	Not tested
DV2 <sup>419-447</sup> peptide <i>Schmidt et al, 2010</i>	N/A	IC <sub>90</sub> 0.3 μM	Yes <i>In vitro</i> (hemifusion and pore formation)	DENV1 (0.1 μM), DENV3 (2 μM), DENV4 (0.7 μM)
3-110-22 <i>Schmidt et al, 2012</i>		IC <sub>90</sub> 740 nM	Yes <i>In vitro</i>	DENV1 (not calc.), DENV3 (not calc.), DENV4 (2.3 μM), Kunjin (not active)

lowered DENV yield ( $IC_{50}$  4  $\mu$ M), although the mechanism of action was not investigated (Yennamalli et al., 2009). A highly potent compound, NITD6, was identified as a potential ligand of the BOG pocket and shown to associate with dengue virions (Wang et al., 2009). NITD6 exhibited an  $EC_{50}$  value of 119 nM in a cell-based immunodetection assay and caused the arrest of dengue virions inside endosomes, suggesting that inhibition occurred at a late step in viral entry. Other studies have used a fusion-from-within assay that measured syncytia formation in insect cells to test whether compounds inhibit viral fusion mediated by the E protein (Kampmann et al., 2009). More recently, studies have used an *in vitro* approach to measure DENV fusion in the presence of inhibitors using purified virus particles and liposomes as target membranes (Poh et al., 2009; Schmidt et al., 2012). One study exploring the activity of stem region peptides measured both DENV pore formation and hemifusion to determine that the peptides inhibited a late step in viral fusion (Schmidt et al., 2010).

Despite the recent number of publications of DENV entry and fusion inhibitors, questions remain. In several cases, the biochemical mechanism(s) by which entry inhibition occurs remains to be addressed. In addition, many of the inhibitors have been modeled to fit in the BOG pocket of the E protein and make contacts with specific E protein amino acids, but high resolution structural data unequivocally establishing the BOG pocket as the binding site are lacking. Likewise, no resistance mutations that could yield clues to a binding location have been published. Thus, the binding location for all molecules remains unclear.

## Current work

The identification of a spectrum of small molecule and peptide inhibitors of DENV entry suggest that entry inhibition may be a viable approach to creating antivirals. While currently described DENV entry inhibitors have not yet advanced to preclinical development, they have been useful as molecular tools to study mechanisms of DENV entry. This study describes our surprising discovery that GNF-2, a small molecule inhibitor of intracellular Abl kinases, inhibited DENV when GNF-2 was pre-incubated with virus inoculum. Using GNF-2 as a scaffold, we performed an SAR study and explored mechanism of action for the identified small molecules. We found that the small molecules act post-attachment of virus to cells and block a late step of viral fusion *in vitro*. Through viral passaging against entry inhibitors, we found a single point mutation in the DI/II region of the E protein, E(M196V), supporting previous results showing this region is important for DENV fusion and consistent with our observation that the compounds inhibit this step of DENV entry. The E(M196V) mutation enhanced both viral particle production and entry in a single-cycle reporter assay as well as rendered the virus particles insensitive to small molecule entry inhibitors. Through a focused alanine mutagenesis screen of this region, we identified two other mutations in this region that may confer resistance against our small molecules, possibly through enhanced entry as well. Overall, this work adds to the growing story of small molecule inhibitors of DENV entry and gives support to the earlier hypothesis that the DI/DII interface in the E protein is a critical area for viral entry.

## REFERENCES:

- Acosta, E.G., Castilla, V., and Damonte, E.B. (2008). Functional entry of dengue virus into *Aedes albopictus* mosquito cells is dependent on clathrin-mediated endocytosis. *Journal of General Virology* 89, 474-484.
- Alvarez, M., Rodriguez-Roche, R., Bernardo, L., Vazquez, S., Morier, L., Gonzalez, D., Castro, O., Kouri, G., Halstead, S.B., and Guzman, M.G. (2006). Dengue Hemorrhagic Fever Caused by Sequential Dengue 1–3 Virus Infections over a Long Time Interval: Havana Epidemic, 2001–2002. *The American Journal of Tropical Medicine and Hygiene* 75, 1113-1117.
- Amarasinghe, A., Kuritsky, J.N., Letson, G.W., and Margolis, H.S. (2011). Dengue virus infectio in Africa. In *Emerging Infectious Diseases* [serial on the Internet] (Centers for Disease Control and Prevention).
- Balmaseda, A., Hammond, S.N., Perez, L., Tellez, Y., Saborio, S.I., Mercado, J.C., Cuadra, R., Rocha, J., Perez, M.A., Silva, S., *et al.* (2006). Serotype-Specific Differences in Clinical Manifestations of Dengue. *The American Journal of Tropical Medicine and Hygiene* 74, 449-456.
- Beasley, D.W.C., and Aaskov, J.G. (2001). Epitopes on the Dengue 1 Virus Envelope Protein Recognized by Neutralizing IgM Monoclonal Antibodies. *Virology* 279, 447-458.
- Butrapet, S., Childers, T., Moss, K.J., Erb, S.M., Luy, B.E., Calvert, A.E., Blair, C.D., Roehrig, J.T., and Huang, C.Y.H. (2011). Amino acid changes within the E protein hinge region that affect dengue virus type 2 infectivity and fusion. *Virology* 413, 118-127.
- Calisher, C.H., Karabatsos, N., Dalrymple, J.M., Shope, R.E., Porterfield, J.S., Westaway, E.G., and Brandt, W.E. (1989). Antigenic Relationships between Flaviviruses as Determined by Cross-neutralization Tests with Polyclonal Antisera. *Journal of General Virology* 70, 37-43.
- Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., and Marks, R.M. (1997). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* 3, 866-871.
- Chu, J.J.H., and Yang, P.L. (2007). c-Src protein kinase inhibitors block assembly and maturation of dengue virus. *Proceedings of the National Academy of Sciences* 104, 3520-3525.
- de Wispelaere, M., and Yang, P.L. (2012). Mutagenesis of the DI/DIII Linker in Dengue Virus Envelope Protein Impairs Viral Particle Assembly. *Journal of Virology* 86, 7072-7083.

Fritz, R., Stiasny, K., and Heinz, F.X. (2008). Identification of specific histidines as pH sensors in flavivirus membrane fusion. *The Journal of Cell Biology* 183, 353-361.

Gollins, S.W., and Porterfield, J.S. (1986). The Uncoating and Infectivity of the Flavivirus West Nile on Interaction with Cells: Effects of pH and Ammonium Chloride. *Journal of General Virology* 67, 1941-1950.

Gubler, D.J. (1976). A simple technique for demonstrating transmission of dengue virus by mosquitoes without the use of vertebrate hosts. *American Journal of Tropical Medicine and Hygiene* 25, 5.

Harrison, S.C. (2005). Mechanism of Membrane Fusion by Viral Envelope Proteins. In *Advances in Virus Research*, R. Polly, ed. (Academic Press), pp. 231-261.

Harrison, S.C. (2008). Viral membrane fusion. *Nature Structural & Molecular Biology* 15, 9.

Holden, K.L., Stein, D.A., Pierson, T.C., Ahmed, A.A., Clyde, K., Iversen, P.L., and Harris, E. (2006). Inhibition of dengue virus translation and RNA synthesis by a morpholino oligomer targeted to the top of the terminal 3' stem-loop structure. *Virology* 344, 439-452.

Hrobowski, Y.M., Garry, R.F., and Michael, S.F. (2005). Peptide inhibitors of dengue virus and West Nile virus infectivity. *Virology Journal* 2.

Hsieh, S.-C., Tsai, W.-Y., and Wang, W.-K. (2010). The Length of and Nonhydrophobic Residues in the Transmembrane Domain of Dengue Virus Envelope Protein Are Critical for Its Retention and Assembly in the Endoplasmic Reticulum. *Journal of Virology* 84, 4782-4797.

Kampmann, T., Yennamalli, R., Campbell, P., Stoermer, M.J., Fairlie, D.P., Kobe, B., and Young, P.R. (2009). In silico screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses. *Antiviral Research* 84, 234-241.

Kilby, J.M., Hopkins, S., Venetta, T.M., DiMassimo, B., Cloud, G.A., Lee, J.Y., Alldredge, L., Hunter, E., Lambert, D., Bolognesi, D., *et al.* (1998). Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nature Medicine* 4, 6.

Kinney, R.M., Huang, C.Y.-H., Rose, B.C., Kroeker, A.D., Dreher, T.W., Iversen, P.L., and Stein, D.A. (2005). Inhibition of Dengue Virus Serotypes 1 to 4 in Vero Cell Cultures with Morpholino Oligomers. *Journal of Virology* 79, 5116-5128.

Konishi, E., and Mason, P.W. (1993). Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. *Journal of Virology* 67, 1672-1675.

Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., *et al.* (2002). Structure of Dengue Virus: Implications for Flavivirus Organization, Maturation, and Fusion. *Cell* 108, 717-725.

Lee, E., Weir, R.C., and Dalgarno, L. (1997). Changes in the Dengue Virus Major Envelope Protein on Passaging and Their Localization on the Three-Dimensional Structure of the Protein. *Virology* 232, 281-290.

Lin, S.-R., Zou, G., Hsieh, S.-C., Qing, M., Tsai, W.-Y., Shi, P.-Y., and Wang, W.-K. (2011). The Helical Domains of the Stem Region of Dengue Virus Envelope Protein Are Involved in both Virus Assembly and Entry. *Journal of Virology* 85, 5159-5171.

Lindenbach, B.D., and Rice, C. (2001). Flaviviridae: The Viruses and Their Replication. *Fundamentals of Virology*, eds Knipe and Howley 1, 589-639.

Mackenzie, J.M., and Westaway, E.G. (2001). Assembly and Maturation of the Flavivirus Kunjin Virus Appear To Occur in the Rough Endoplasmic Reticulum and along the Secretory Pathway, Respectively. *Journal of Virology* 75, 10787-10799.

Mackenzie, J.S., Gubler, D.J., and Petersen, L.R. (2004). Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nature Medicine* 10, 12.

Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America* 100, 6986-6991.

Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature* 427, 313-319.

Moesker, B., Rodenhuis-Zybert, I.A., Meijerhof, T., Wilschut, J., and Smit, J.M. (2010). Characterization of the functional requirements of West Nile virus membrane fusion. *Journal of General Virology* 91, 389-393.

Mukhopadhyay, S., Kuhn, R.J., and Rossmann, M.G. (2005). A structural perspective of the flavivirus life cycle. *Nat Rev Micro* 3, 13-22.

Nelson, S., Poddar, S., Lin, T.-Y., and Pierson, T.C. (2009). Protonation of Individual Histidine Residues Is Not Required for the pH-Dependent Entry of West Nile Virus: Evaluation of the “Histidine Switch” Hypothesis. *Journal of Virology* 83, 12631-12635.

Peiris, J., and Porterfield, J. (1979). Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines. *Nature* 282, 3.

- Poh, M.K., Yip, A., Zhang, S., Priestle, J.P., Ma, N.L., Smit, J.M., Wilschut, J., Shi, P.-Y., Wenk, M.R., and Schul, W. (2009). A small molecule fusion inhibitor of dengue virus. *Antiviral Research* 84, 260-266.
- Rosen, L. (1987). Sexual transmission of dengue viruses by *Aedes albopictus*. *American Journal of Tropical Medicine and Hygiene* 37, 5.
- Sánchez-San Martín, C., Sosa, H., and Kielian, M. (2008). A Stable Prefusion Intermediate of the Alphavirus Fusion Protein Reveals Critical Features of Class II Membrane Fusion. *Cell Host & Microbe* 4, 600-608.
- Schmidt, A.G., Lee, K., Yang, P.L., and Harrison, S.C. (2012). Small-Molecule Inhibitors of Dengue-Virus Entry. *PLoS Pathog* 8, e1002627.
- Schmidt, A.G., Yang, P.L., and Harrison, S.C. (2010). Peptide Inhibitors of Dengue-Virus Entry Target a Late-Stage Fusion Intermediate. *PLoS Pathog* 6, e1000851.
- Stadler, K., Allison, S.L., Schalich, J., and Heinz, F.X. (1997). Proteolytic activation of tick-borne encephalitis virus by furin. *Journal of Virology* 71, 8475-8481.
- Stiasny, K., Allison, S.L., Marchler-Bauer, A., Kunz, C., and Heinz, F.X. (1996). Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. *Journal of Virology* 70, 8142-8147.
- Tassaneetrithep, B., Burgess, T.H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M.A., Pattanapanyasat, K., Sarasombath, S., Birx, D.L., *et al.* (2003). DC-SIGN (CD209) Mediates Dengue Virus Infection of Human Dendritic Cells. *The Journal of Experimental Medicine* 197, 7.
- van der Schaar, H.M., Rust, M.J., Chen, C., van der Ende-Metselaar, H., Wilschut, J., Zhuang, X., and Smit, J.M. (2008). Dissecting the Cell Entry Pathway of Dengue Virus by Single-Particle Tracking in Living Cells. *PLoS Pathog* 4, e1000244.
- Vaughn, D.W., Green, S., Kalayanarooj, S., Innis, B.L., Nimmannitya, S., Suntayakorn, S., Endy, T.P., Raengsakulrach, B., Rothman, A.L., Ennis, F.A., *et al.* (2000). Dengue Viremia Titer, Antibody Response Pattern, and Virus Serotype Correlate with Disease Severity. *Journal of Infectious Diseases* 181, 2-9.
- Wang, Q.-Y., Patel, S.J., Vangrevelinghe, E., Xu, H.Y., Rao, R., Jaber, D., Schul, W., Gu, F., Heudi, O., Ma, N.L., *et al.* (2009). A Small Molecule Dengue Virus Entry Inhibitor. *Antimicrob Agents Chemother*, AAC.01148-01108.
- Westaway, E.G., Mackenzie, J.M., Kenney, M.T., Jones, M.K., and Khromykh, A.A. (1997). Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. *Journal of Virology* 71, 6650-6661.

WHO (2012). Dengue and severe dengue.

Yennamalli, R., Subbarao, N., Kampmann, T., McGeary, R., Young, P., and Kobe, B. (2009). Identification of novel target sites and an inhibitor of the dengue virus E protein. *Journal of Computer-Aided Molecular Design*.

Yu, I.M., Zhang, W., Holdaway, H.A., Li, L., Kostyuchenko, V.A., Chipman, P.R., Kuhn, R.J., Rossmann, M.G., and Chen, J. (2008). Structure of the Immature Dengue Virus at Low pH Primes Proteolytic Maturation. *Science* *319*, 1834-1837.

Zhang, W., Chipman, P.R., Corver, J., Johnson, P.R., Zhang, Y., Mukhopadhyay, S., Baker, T.S., Strauss, J.H., Rossmann, M.G., and Kuhn, R.J. (2003a). Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. *Nature Structural & Molecular Biology* *10*, 6.

Zhang, Y., Corver, J., Chipman, P.R., Zhang, W., Pletnev, S.V., Sedlak, D., Baker, T.S., Strauss, J.H., Kuhn, R.J., and Rossmann, M.G. (2003b). Structures of immature flavivirus particles. *EMBO* *22*, 10.

Zhang, Y., Zhang, W., Ogata, S., Clements, D., Strauss, J.H., Baker, T.S., Kuhn, R.J., and Rossmann, M.G. (2004). Conformational Changes of the Flavivirus E Glycoprotein. *Structure* *12*, 1607-1618.

Zhou, Z., Khaliq, M., Suk, J.-E., Patkar, C., Li, L., Kuhn, R.J., and Post, C.B. (2008). Antiviral Compounds Discovered by Virtual Screening of Small Molecule Libraries against Dengue Virus E Protein. *ACS Chemical Biology* *3*, 765-775.



**Chapter 2:** Characterization of GNF-2, a small molecule inhibitor of cellular Abl kinases, as an inhibitor of dengue virus entry

**Acknowledgments:**

The initial cell-based screen of small molecule kinase inhibitors that led to this project was performed by Dr. Justin Chu during his time in the Yang lab. The fluorescence polarization assay with recombinant dengue envelope protein was performed by Aaron Schmidt in Dr. Stephen Harrison's lab. All GNF-2 analogs, both conjugated and those tested as part of the structure-activity relationship study, were synthesized by Drs. Chandra Miduturu, Jinhua Wang, and Xianming Deng, members of Dr. Nathanael Gray's lab. The activity assay for GNF-2 analogs in Bcr-Abl transformed Ba/F3 cells was performed by Dr. Jianming Zhang in the Gray lab. I would also like to thank members of the Yang lab, past and present, for helpful discussions of my project as it unfolded.

## SUMMARY

In this chapter, we found that GNF-2, a previously characterized small molecule inhibitor of cellular Bcr-Abl kinase, lowers dengue virus (DENV) titer when present at two separate times during viral infection. We focused on characterizing the inhibition of DENV2 when GNF-2 is pre-incubated with virus inoculum and present only during the first hour of target cell infection, an effect that could not be recapitulated with another small molecule Abl kinase inhibitor. We found that fluorophore-conjugated GNF-2 co-localized with DENV2 envelope (E) protein inside cells shortly after infection, and that a biotinylated analog of GNF-2 bound directly to purified dengue virions *in vitro*. Using fluorescent polarization assays and recombinant soluble envelope (E) protein, we found that a FITC-conjugated GNF-2 derivative interacts with the pre-fusion dimer conformation of E. Initially, we used a cell-impermeable analog of GNF-2, c-GNF-2, to confirm that inhibition of DENV2 infectivity is independent of intracellular Abl kinases. Since c-GNF-2 was over three times less effective at reducing DENV2 infectivity, we performed a focused medicinal chemistry study to identify other disubstituted pyrimidines with increased potency compared to GNF-2's activity in the cellular infectivity assay. We tested these molecules against other DENV serotypes and found that they had wide ranges of activities. Based on the interaction of GNF-2 analogs with recombinant E protein and purified virions as well as the range of activities against DENV serotypes, we hypothesize that this class of compounds inhibits DENV entry via a direct interaction with the DENV envelope protein on the surface of the virion.

## INTRODUCTION

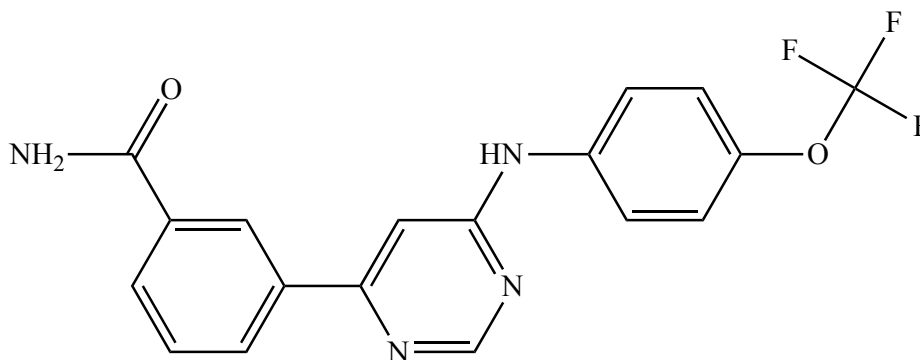
Dengue virus (DENV) is a mosquito-borne virus that causes an estimated annual 50 million cases of dengue fever worldwide (WHO, 2012). Despite its status as an international health concern, much remains unknown about the DENV life cycle. The DENV genome encodes only ten viral proteins, making the virus highly dependent on a variety of cellular proteins and processes for a successful, productive infection. In an attempt to identify cellular kinases that were necessary for successful DENV infection, our lab performed a cell-based microscopy screen of small molecule inhibitors that were previously published as cellular kinase inhibitors (Chu and Yang, 2007).

Several small molecules were identified that significantly reduced DENV infection. When the kinase selectivities of these compounds were examined, we found six of the molecules were known to inhibit the Abl kinase family (Table 2-1). Four of these six molecules also have documented activity against Src family kinases (Hennequin et al., 2006; Holen et al., 1995; Okram et al., 2006; Shah et al., 2004), while one, imatinib, is known to inhibit c-Kit, PDGFR, and DDR1/2 (Buchdunger et al., 1996). However, the last compound, GNF-2, is an allosteric kinase inhibitor and known to be highly selective, exhibiting measurable activity against only Abl family kinases when screened against a panel of 450 kinases (Adrian et al., 2006; Zhang et al., 2010). In order to explore the role of Abl kinases in DENV infection, we performed additional experiments with imatinib and GNF-2, which have similar measured activity against Abl kinases in biochemical and cellular assays (Figure 2-1), but work via different molecular mechanisms. Imatinib is an active site inhibitor that traps Abl in the 'DFG-out' conformation, while GNF-2 is an

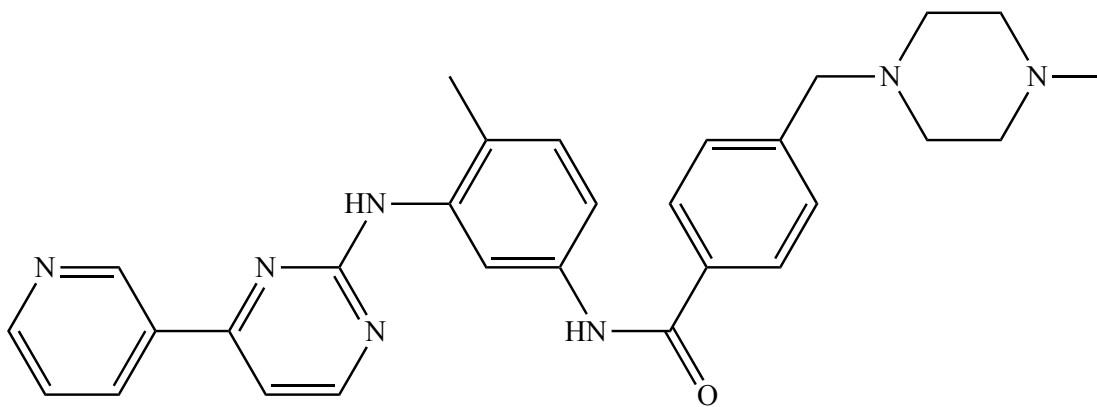
**Table 2-1. Small molecule protein kinase inhibitors that inhibit DENV infection, as monitored by an immunofluorescence image-based screen. *Adapted from (Chu and Yang, 2007)***

<b>Inhibitor</b>	<b>Src</b>	<b>Abl</b>	<b>c-Kit, PDGFR, VEGFR</b>	<b>Other kinase targets</b>
K002				CDKs
K014 (imatinib)		X	X	
K039				c-Raf
K040				JAK1, -2, -3
K003	X	X	X	
K013 (GNF-2)		X		
K030	X			Kdr
K032				CK II
K117	X			
K045 (AZD0530)	X	X		
K005 (Dasatinib)	X	X	X	
K025 (SU11652)			X	FGFR
K028 (Lavendustin A)	X			EGFR
K026 (SU5271)				EGFR
K144 (Kenpaullone)	X			CDKs, GSK3-b
K115 (Lavendustin C)	X			CaMK II
K116 (MC7)				MLCK
K118 (Tyrphostin46)	X	X	X	Multi-targeted

GNF-2, IC<sub>50</sub> (kinase) 138 ± 5 nM



Imatinib, IC<sub>50</sub> (kinase) 190 ± 6 nM



**Figure 2-1. Structures of (top) GNF-2 and (bottom) imatinib.** IC<sub>50</sub> values shown are *in vitro* against Abl family kinases. (Adrian et al., 2006; Buchdunger et al., 1996)

allosteric inhibitor of Abl kinase that binds in a hydrophobic myristoyl-binding pocket located near the C-terminus of the kinase domain.

In order to determine when in the viral infectious cycle these compounds exhibit anti-DENV activity, we performed order of addition experiments and discovered that GNF-2, but not imatinib, inhibited DENV infection when present during two separate times during viral infection. While both small molecules inhibited DENV when added to cells after initial infection, only GNF-2 lowered DENV yield when pre-incubated with virus inoculum. In this chapter, we describe our characterization of GNF-2's inhibition of DENV infectivity when present prior to and during initial cellular infection. We applied both cellular and biochemical approaches to explore how GNF-2 inhibits DENV infectivity, and collectively our findings led to the hypothesis that GNF-2 interacts with DENV envelope protein on the surface of the virion and affects entry. This hypothesis was corroborated by our discovery, made in collaboration with the Gray laboratory, of GNF-2 analogs that lack Abl kinase inhibitory activity yet have greater potency than GNF-2 when present during initial DENV infection. Collectively, these results identified GNF-2 and its analogs as inhibitors of DENV infectivity via interactions with the DENV E protein and provide chemical tools to investigate mechanism(s) of action in future experiments.

## **RESULTS**

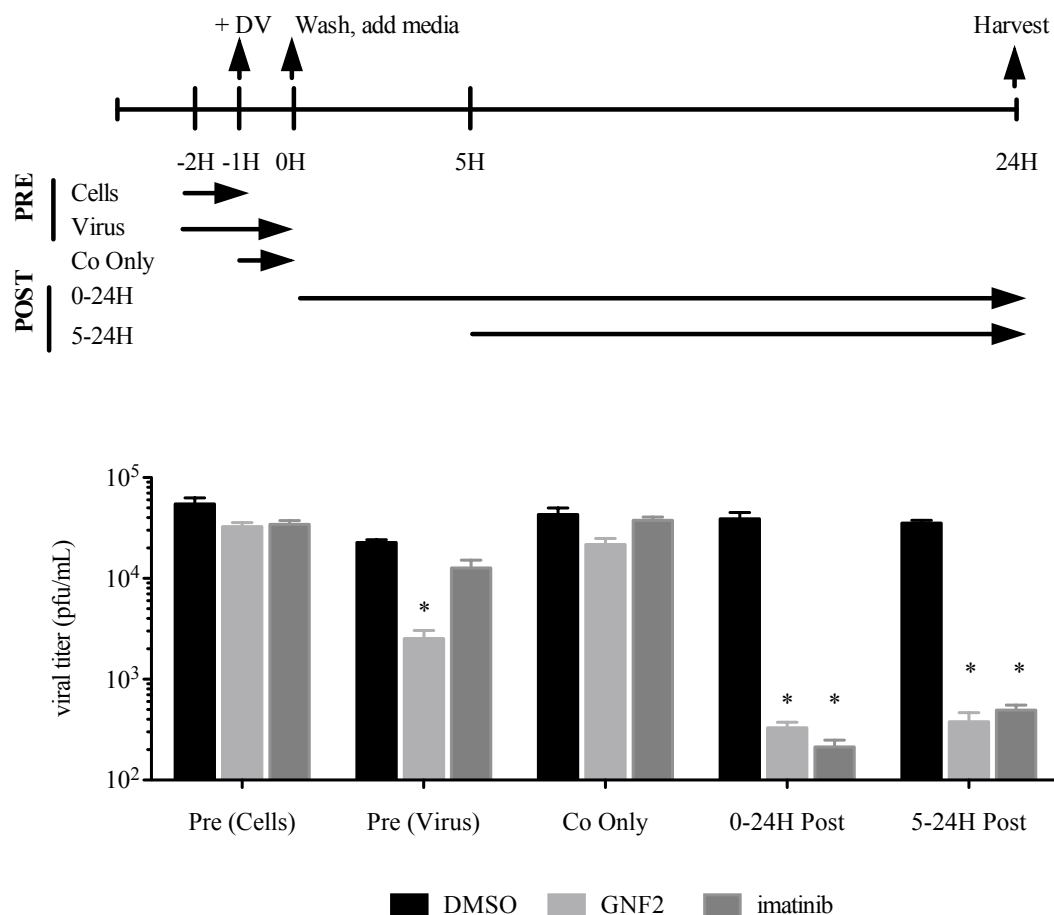
### **GNF-2 acts at two separate parts of the DENV life cycle**

We initially performed order of addition experiments with GNF-2 and imatinib to determine the point(s) in the DENV life cycle during which the small molecules exert

their effect(s). With compounds present at various times during infection (Figure 2-2), we infected cells at an MOI of 1 and determined DENV yield by plaque-forming assay (PFA) at twenty-four hours post infection, corresponding to approximately one complete life cycle of DENV (Lambeth et al., 2005). We found that GNF-2 and imatinib both led to a significant reduction in DENV yield when added directly after viral infection, and addition of either inhibitor five hours post-infection caused a similar magnitude of inhibition (Figure 2-2). The average time between surface binding of virions and endosomal fusion for DENV has been shown to be 12.5 minutes (van der Schaar et al., 2007), strongly suggesting that the small molecules are affecting events downstream of viral entry in the DENV life cycle. Our current hypothesis is that this post-entry inhibition is due to inhibition of host Abl kinases, since it is observed at a similar magnitude with imatinib and GNF-2. Surprisingly, we also observed a significant decrease in DENV yield when GNF-2 was pre-incubated with the viral inoculum prior to infection. This anti-DENV activity was not observed when the inoculum was pre-incubated with imatinib, suggesting that it may be due to an Abl-independent mechanism. In addition, no reduction in DENV yield was observed when GNF-2 was pre-incubated with cells or only present during the one hour initial infection, demonstrating that pre-incubation of the compound with virus was a critical step for inhibiting DENV. This led us to hypothesize that the observed DENV inhibition is due to an interaction of GNF-2 with a target present in the virus inoculum.

To further illustrate that GNF-2 inhibits DENV at two separate steps in the viral life cycle, one mediated by Abl kinases and one mediated by an independent target, we performed additivity experiments with GNF-2, imatinib, and NITD6 (Figure 2-3), a

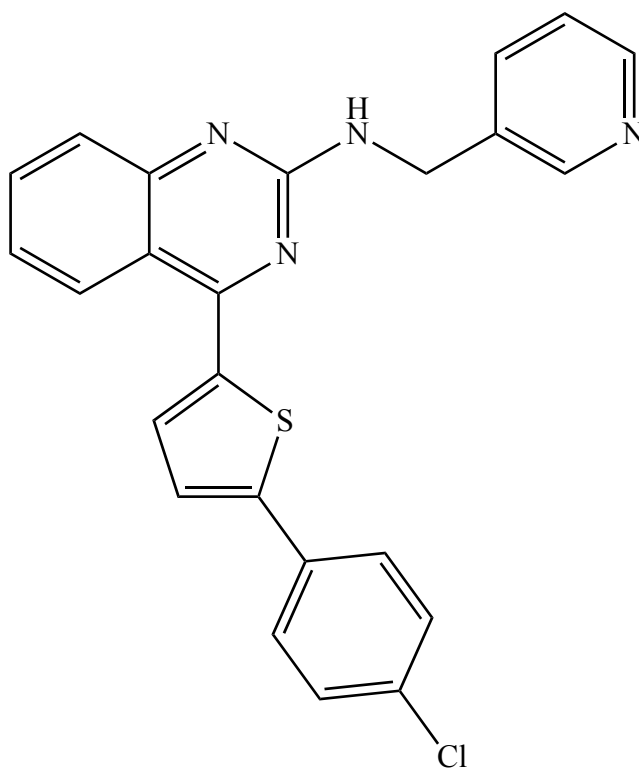




**Figure 2-2. GNF-2 lowers DENV titer when pre-incubated with virus inoculum.**

Order of addition experiments with GNF-2 and imatinib. Compounds were tested at 15  $\mu$ M and BHK-21 cells were infected at a multiplicity of infection (MOI) of 1. DENV yield in supernatant was determined by plaque-forming assay at 24 hours post-infection. Each bar represents the mean of three replicates with error bars showing standard deviation. Student's t test, \* indicates p value < 0.01\*

NITD6, IC<sub>50</sub> 198 nM in A549 cells against DENV2

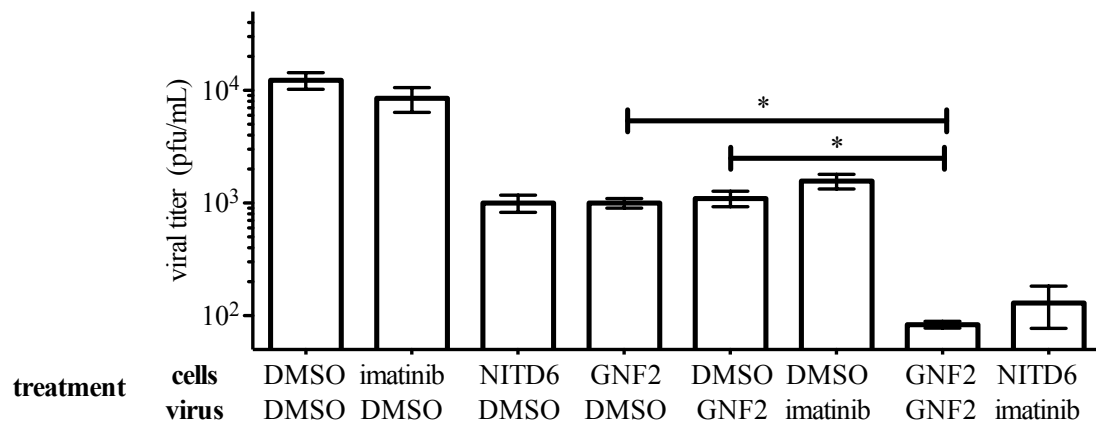


**Figure 2-3. Structure of NITD6**, a previously published DENV small molecule entry inhibitor (Wang et al., 2009)

previously validated inhibitor of DENV entry shown to interact with the DENV envelope protein (Wang et al., 2009). EC<sub>90</sub> values for each compound were determined in dose-response titration experiments. GNF-2 and imatinib were both found to have EC<sub>90</sub> values of 8  $\mu$ M when added to cells post-infection. When compounds were incubated with virus inocula at 37°C for 45 minutes prior to addition to cells, NITD6 had a calculated EC<sub>90</sub> value when present during entry of 200 nM, while GNF-2's EC<sub>90</sub> during entry was 18  $\mu$ M.

Pre-treatment of the virus inoculum with GNF-2 or NITD6 significantly reduced DENV titer measured at 24 hours post-infection (Figure 2-4), while post-treatment of infected cells with equal molar concentrations of GNF-2 or imatinib also showed significant inhibition of DENV yield. Combining pre-treatment of the inoculum with NITD6 and post-treatment of cells with imatinib had an additive effect on DENV yield, consistent with inhibition of DENV via two separate mechanisms. Importantly, an additive effect was also observed when GNF-2 pre-treatment of the inoculum was combined with GNF-2 post-infection treatment of DENV-infected cells, showing that GNF-2 alone, when present at two separate times during infection, can recapitulate the combined effects of NITD6 and imatinib.

Together, these data suggest that GNF-2 acts at two separate stages of the DENV life cycle: first, blocking DENV entry into cells via a target present in the virus inoculum, and second, inhibiting DENV at a post-entry step mediated by a target shared by imatinib and GNF-2, presumably Abl kinases. GNF-2's activity as an intracellular Abl kinase inhibitor has been described previously (Adrian et al., 2006), and the role of Abl kinases in DENV infection post-entry is currently under investigation by others in our laboratory.



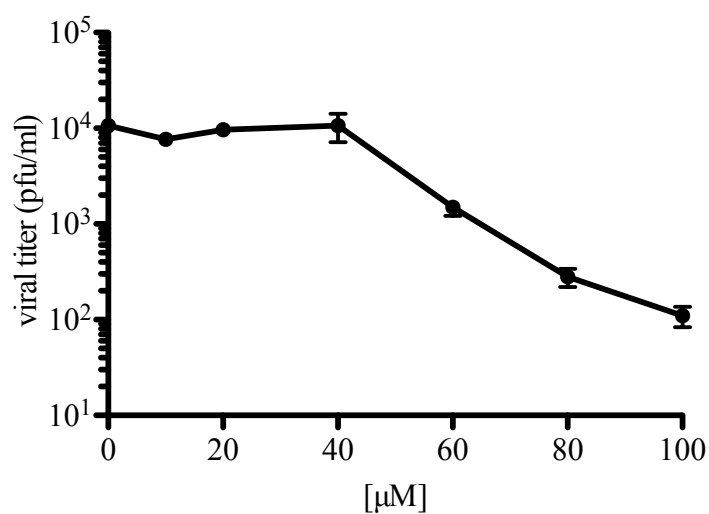
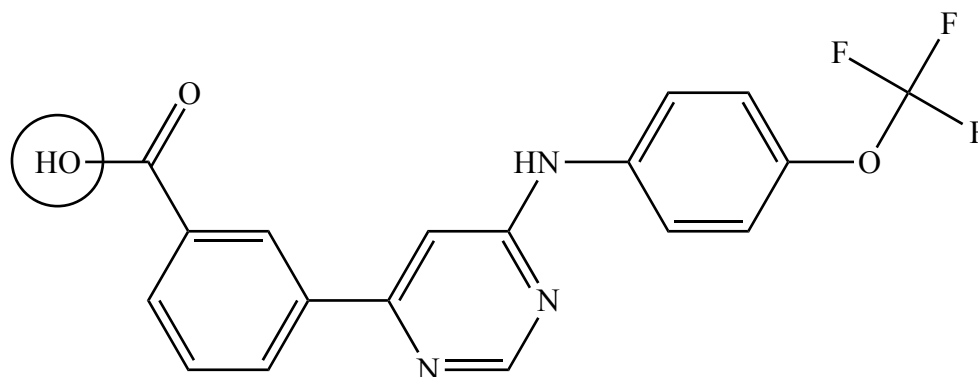
**Figure 2-4. GNF-2 has additive effects on DENV titer.** All virus and cell treatments were done at EC<sub>90</sub> values determined empirically. Virus treatment was carried out for 45 minutes at 37°C prior to initial cell infection (MOI 1). Treatment of BHK-21 cells was begun immediately after the initial one-hour infection. Each bar represents the mean of three replicates with error bars showing standard deviation. \* indicates p value < 0.01

In this project, we focused on characterizing the previously unknown Abl-independent activity of GNF-2 as an inhibitor of DENV infectivity.

### **A cell impermeable analog of GNF2 retains anti-DENV activity**

In previous studies, GNF-2 has been screened for activity against more than 450 kinases and found to be a highly selective inhibitor of Abl family kinases (Adrian et al., 2006). This fact, combined with our result that GNF-2 must be pre-incubated with the virus inoculum to cause a significant decrease in DENV yield, led us to the hypothesis that GNF-2 acts on an extracellular target present in the virus inoculum. We chose to test this hypothesis by using a cell impermeable analog in which the carboxamide functionality of GNF-2 was replaced with a carboxylate (c-GNF-2; Figure 2-5). The activity of c-GNF-2 against c-Abl kinase in a biochemical kinase assay is comparable to that observed with GNF-2 (Choi et al., 2009); however, c-GNF2 was unable to inhibit the proliferation of Bcr-Abl transformed Ba/F3 cells ( $IC_{50} > 10 \mu M$  versus  $IC_{50}$  140 nM for GNF-2), confirming its inability to penetrate the plasma membrane and access the intracellular kinase target. In order to assess c-GNF-2's ability to inhibit DENV infection, we pre-incubated the viral inoculum with c-GNF-2 (20-100  $\mu M$ , 45 minutes, 37 °C) and added the mixture to cells for the initial hour infection, the same conditions used to determine the  $EC_{90}$  value during entry of GNF-2. Supernatants were collected at 24 hours post-infection and yield of infectious virus present was measured by PFA. Under these conditions, we observed a one-log drop in DENV at approximately 60  $\mu M$  (Figure 2-5), showing that c-GNF-2 inhibits DENV infectivity, despite its inability to penetrate the

c-GNF-2

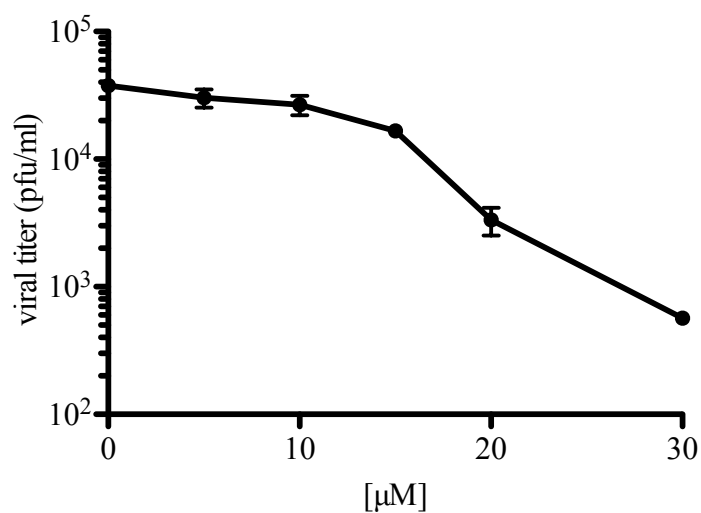
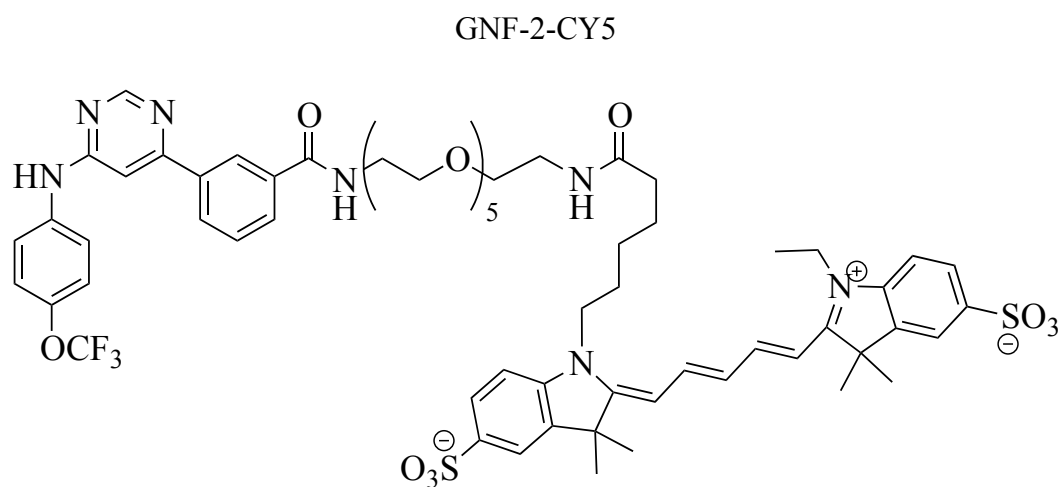


**Figure 2-5. c-GNF-2 structure and dose-response titration against DENV2.** The change to a carboxyl from GNF-2 is circled on the structure. c-GNF-2 was pre-incubated with virus inocula for 45 minutes at 37°C before infection of BHK-21 cells (MOI 1). Each point represents the mean of three replicates with error bars showing standard deviation. EC<sub>90</sub> during entry of c-GNF-2 ~60 μM.

cellular membrane. This result supports our initial hypothesis that GNF-2 has an extracellular target present in the virus inoculum.

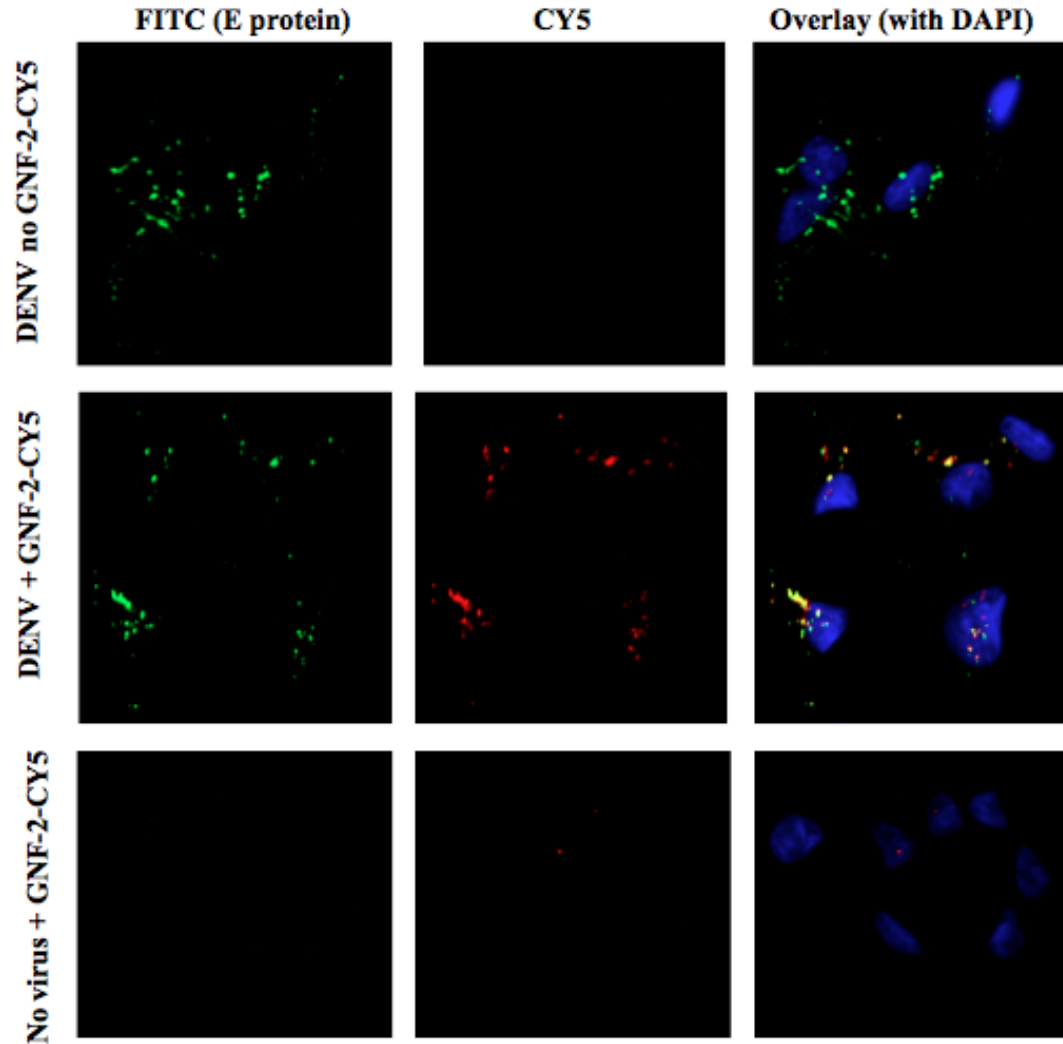
### **CY5-GNF-2 co-localizes with dengue virions after virion uptake**

We hypothesized that GNF-2 may target the virion itself as a way of inhibiting DENV infectivity. To examine the location of GNF-2 during viral entry into cells, we synthesized a derivative of GNF-2 conjugated to the fluorescent dye CY5. The resulting compound, CY5-GNF-2 (Figure 2-6), inhibited DENV in cellular assays with an  $EC_{90}$  value during entry ( $\sim 20 \mu\text{M}$ ) similar to that of GNF-2. DENV was incubated with  $25 \mu\text{M}$  GNF-2-CY5 before infecting cells (MOI 10) for 30 minutes at  $37^\circ\text{C}$ , conditions that were empirically determined to permit binding and internalization of virions. Unbound compound and virus were removed by extensive washing and cells were fixed to permit detection of the DENV envelope (E) protein by immunofluorescence. DENV was visible inside cells as punctate staining (Figure 2-7, top row). GNF-2-CY5 was observed inside cells when the compound was pre-incubated with DENV inoculum, and the majority of this signal co-localized with the E protein (Figure 2-7; middle row). In the absence of DENV, little GNF-2-CY5 was observed inside cells (Figure 2-7, bottom row), demonstrating the inability of this compound to penetrate the plasma membrane and the efficient removal of extracellular compound during wash steps. The uptake of GNF-2-CY5, moreover, appears to be DENV-specific since it was not observed with a control virus, vesicular stomatitis virus (VSV) (Figure 2-8), whose yield was unaffected by pre-incubation with GNF-2 (data not shown).

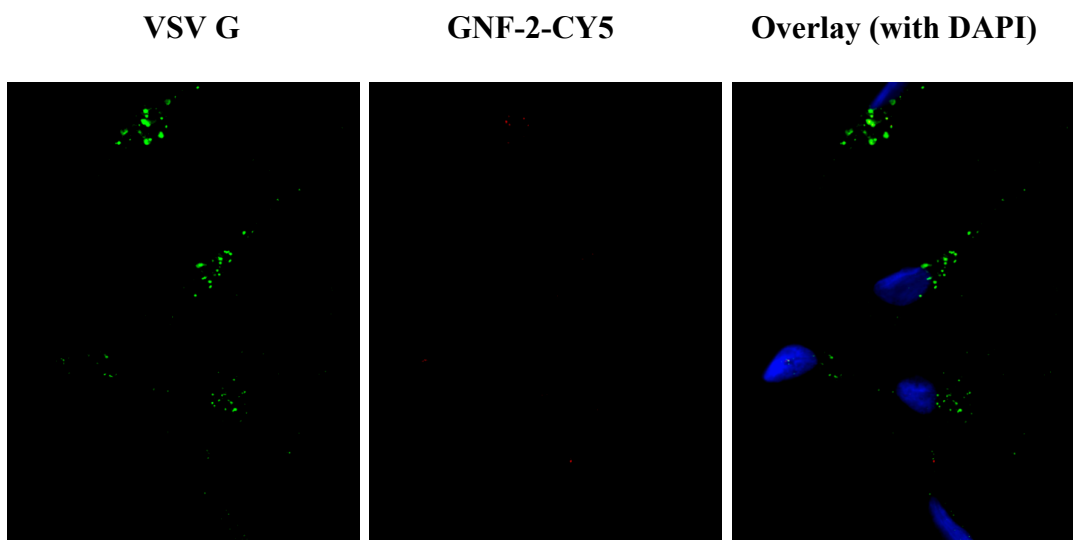


**Figure 2-6. GNF-2-CY5 structure and dose-response titration against DENV2.** GNF-2-CY5 was pre-incubated with virus inocula for 45 minutes at 37°C before infection of BHK-21 cells (MOI 1). EC<sub>90</sub> value during entry ~20 μM. Each point represents the mean of three replicates with error bars showing standard deviation.





**Figure 2-7. GNF-2 co-localizes with DENV E protein.** DENV was incubated with 20  $\mu$ M GNF-2-CY5 prior to infection of cells (MOI 10). Cells were infected for 30 minutes at 37°C before fixing and staining for the DENV E protein. (*Top row*) Cells infected with DENV. (*Middle row*) Cells treated with DENV pre-incubated with GNF-2-CY5. (*Bottom row*) Cells treated with GNF-2-CY5 only. Images are representative of a field of view from 2 separate experiments.



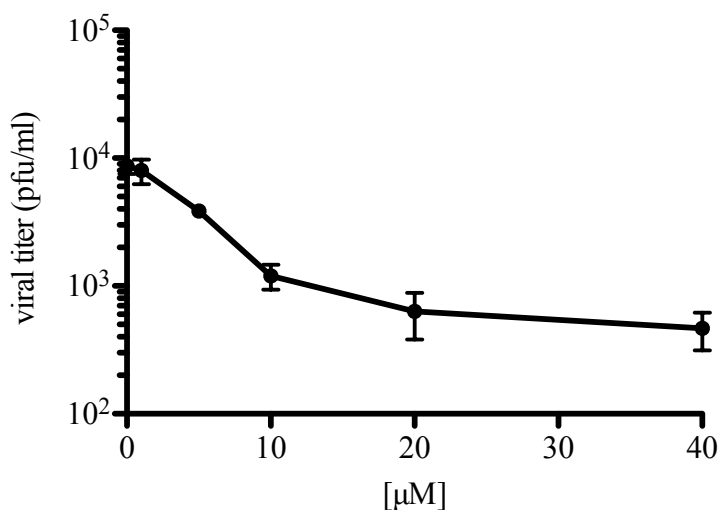
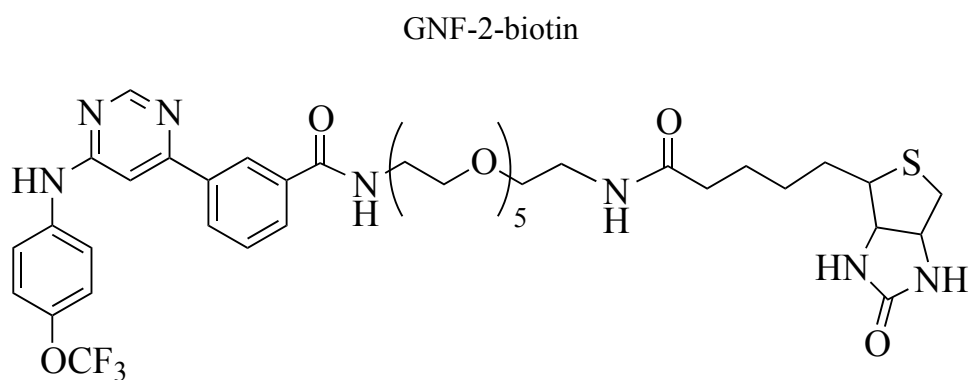
**Figure 2-8. GNF-2-CY5 is not taken up in presence of VSV.** Vesicular stomatitis virus (VSV) was pre-incubated with 20  $\mu$ M GNF-2-CY5 at 37°C. Cells were fixed 30 minutes post-infection (MOI 10) and stained for VSV G protein. Images are representative of a field of view.

## **GNF-2 interacts directly with the dengue virus envelope protein**

The fact that the anti-DENV effects of both GNF-2 and c-GNF-2 require pre-incubation with virus inocula prior to infection coupled with the co-localization of GNF-2-CY5 with DENV E protein inside cells led us to hypothesize that GNF-2 targets the dengue virion. To explore this hypothesis, we tested whether a biotin-conjugated derivative of GNF-2 (GNF-2-biotin, Figure 2-9) could bind and capture purified DENV2 virions. GNF-2-biotin was determined to have an  $EC_{90}$  value during entry in cellular assays ( $\sim 18 \mu\text{M}$ ) similar to that of the parental GNF-2.

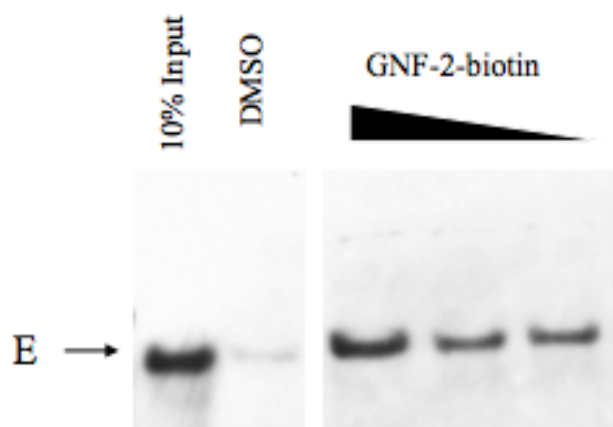
Dengue virions were harvested from cell culture supernatant, concentrated by PEG precipitation, purified over a potassium tartrate gradient, and dialyzed in TAN buffer prior to quantification and incubation with GNF-2-biotin at  $37^\circ$  for 45 minutes. Streptavidin beads were used to bind GNF-2-biotin and washed ten times to remove any unbound DENV. The beads were boiled in SDS buffer and the released proteins were analyzed by Western blot for the DENV E protein. We found that GNF-2-biotin captures purified DENV2 virions in a dose-dependent manner (Figure 2-10).

Knowing that GNF-2 directly interacts with the dengue virion, we next wanted to investigate how this interaction between small molecule and virion occurs. The main protein component on the dengue virion surface is the envelope (E) protein, which on the mature virion associates as a homodimer and tightly packs in a herringbone arrangement (Kuhn et al., 2002). It is possible that GNF-2 and conjugated derivatives could bind to the virion via non-specific interactions with the lipid bilayer, but we hypothesized that the small molecules have a direct interaction with the E protein. To test this, our collaborator in Dr. Stephen Harrison's lab used a fluorescence polarization assay to monitor whether a



**Figure 2-9. GNF-2-biotin structure and dose-response titration against DENV2.** =

GNF-2-biotin was pre-incubated with virus inocula for 45 minutes at 37°C before infection of BHK-21 cells (MOI 1). EC<sub>90</sub> value during entry ~18 μM. Each point represents the mean of three replicates with error bars showing standard deviation.



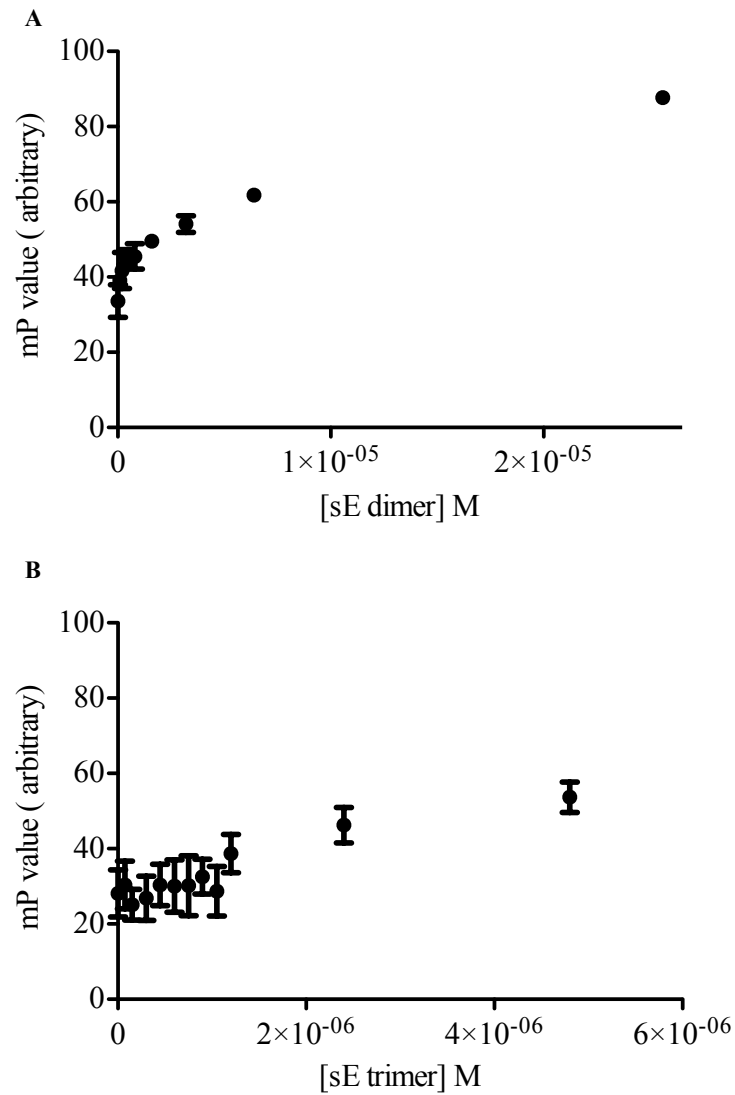
**Figure 2-10. GNF-2-biotin interacts with purified DENV2.** DENV was precipitated and purified over a potassium tartrate gradient before being incubated with GNF-2-biotin for 45 minutes at 37°C. GNF-2-biotin concentrations: 10, 5, 1  $\mu$ M. Streptavidin resin was used to bind GNF-2-biotin and washed ten times with HNE buffer to remove any unbound virus. Samples were boiled and run on a SDS-PAGE gel and analyzed by Western blotting for the DENV E protein using 4G2, an antibody that recognizes the fusion loop.

FITC-conjugated GNF2 analog (GNF-2-FITC) interacts with a recombinant, soluble form of the DENV2 E protein comprised of domains I, II, and III without the stem region, which exists as a pre-fusion dimer in aqueous solution (sE dimer). Incubation of GNF-2-FITC with increasing concentrations of DENV2 sE dimer led to a decrease in fluorescence anisotropy (Figure 2-11A), suggesting a physical interaction between GNF-2-FITC and the pre-fusion DENV2 sE dimer. We also tested whether GNF-2-FITC interacted with the DENV2 post-fusion trimer conformation of sE, and found no change in fluorescence polarization (Figure 2-11B). These data suggest that the conformation of sE affects the affinity of GNF-2-FITC for the protein.

Taken together, the fluorescence microscopy and *in vitro* interaction data suggest that GNF-2 affects DENV infectivity by binding to the dengue virion, more specifically the envelope protein dimer on the virion surface. The envelope protein is responsible for all major events of virus entry (binding/attachment, uptake, membrane fusion), making it possible that GNF-2 binding could affect any of these events.

### **GNF-2 has variable inhibitory activity against the DENV serotypes**

Our work up to this point exclusively utilized the DENV2 New Guinea C (NGC) strain, so we next asked if GNF-2 could inhibit entry of other DENV serotypes. DENV serotypes are defined by differences in the amino acid sequences of the E protein (Lindenbach and Rice, 2001), and since we hypothesized that E is the target of GNF-2, we reasoned that we might observe variable activity of GNF-2 against other DENV serotypes. We performed dose-response titration experiments to determine the effect of GNF-2 on the infectivity of strains representative of the other three DENV serotypes.



**Figure 2-11. GNF-2-FITC interacts with recombinant soluble DENV2 E protein**

Fluorescence polarization assay with GNF-2-FITC and soluble recombinant DENV2 E protein in (A) pre-fusion dimer or (B) post-fusion trimer conformation. Points represent the average of two runs, each in triplicate, with errors bars showing standard deviation.

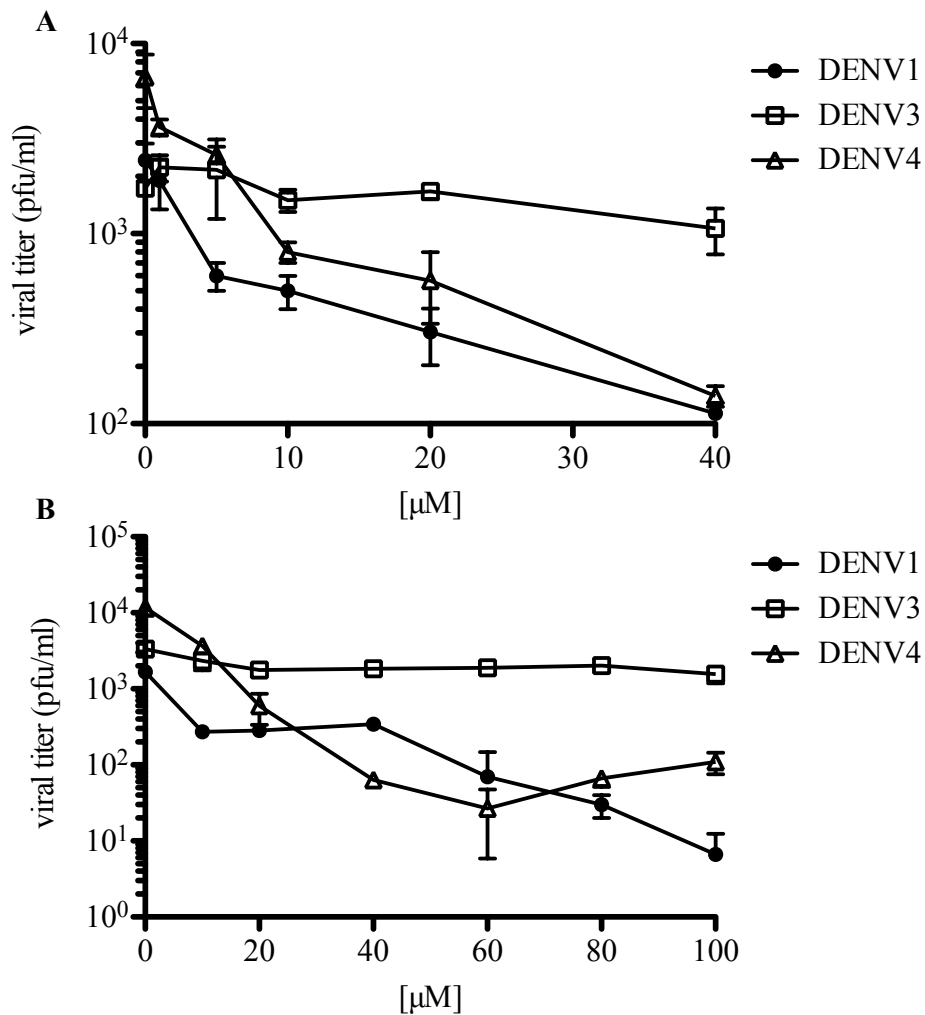
GNF-2 was pre-incubated with virus inocula (MOI 1) for 45 minutes at 37°C, the same experimental setup used to determine the EC<sub>90</sub> value of GNF-2 and its analogs (Figure 2-12). DENV1 strain WP74 (EC<sub>90</sub> value ~20 µM) and DENV4 strain TVP360 (EC<sub>90</sub> value ~18 µM) exhibited sensitivity to GNF-2 comparable to the previously determined value for DENV2 NGC. In contrast, the EC<sub>90</sub> value for the DENV3 strain THD3 was greater than 40 µM, the highest GNF-2 concentration tested, which only caused a two-fold reduction in DENV3 yield. Results with c-GNF-2 showed an even wider range of activities. c-GNF-2 had an EC<sub>90</sub> value of ~20 µM against DENV4, three times more potent than the EC<sub>90</sub> value against DENV2. The EC<sub>90</sub> value against DENV1 (~60 µM) was similar to our previous value against DENV2, while DENV3 THD3 again showed only a two-fold decrease in viral yield at the highest c-GNF-2 concentration tested (100 µM).

Despite the high level of structural homology among DENV serotypes that is evident in high-resolution crystal structures (Modis et al., 2003, 2005), there is only 66% conservation of amino acid sequences among the E proteins (Lindenbach and Rice, 2001). The variable anti-DENV activity of GNF-2 and c-GNF-2 is consistent with our observation that the target of GNF-2 is the E protein.

### **Identification of Abl-inactive GNF-2 analogs that inhibit DENV entry**

Our work to this point focused on characterizing GNF-2's inhibition of DENV entry, separate from the activity of GNF-2 against intracellular Abl kinases. To do this, we used analogs of GNF-2 that were cell-impermeable by virtue of the addition of charge, as for c-GNF-2, or conjugation to fluorophores and biotin, as for GNF-2-CY5,





**Figure 2-12. Dose-response titration of GNF-2 and c-GNF-2 against DENV1, 3, and 4 strains.** All DENV strains were diluted to infect BHK-21 cells at an MOI of 1 and pre-treated with (A) GNF-2 or (B) c-GNF-2 for 45 minutes at 37°C at noted concentrations. Each point represents the mean of three replicates with error bars showing standard deviation. GNF-2 EC<sub>90</sub> values: DENV1 ~20 μM; DENV3 N/A; DENV4 ~18 μM. c-GNF-2 EC<sub>90</sub> values: DENV1 ~60 μM; DENV3 N/A; DENV4 ~20 μM

GNF-2-FITC, and GNF-2-biotin. Despite being cell-impermeable and thus unable to access intracellular Abl kinases, these latter molecules still inhibited DENV2 infectivity with potencies comparable to that of the parental compound, GNF-2, in cellular assays. We sought to use medicinal chemistry to more explicitly determine the structure-activity relationships governing GNF-2's effects on Abl kinases versus its effects on DENV infectivity. Specifically, we wanted to identify compounds that inhibit DENV entry but are devoid of activity against cellular Abl kinases.

Towards this end, a focused structure-activity relationship study was performed to vary the substitution pattern and the substituents on the central pyrimidine core. All compounds were designed and synthesized by members of Dr. Nathanael Gray's lab. A total of 112 compounds were initially tested in our pre-incubation assay (37°C, 45 minutes) at 75  $\mu$ M; compounds that showed a minimum of a one-log drop in DENV2 yield were re-tested at 25  $\mu$ M. Compounds that maintained a one-log drop at this concentration were then tested at six concentrations in the pre-incubation assay against all four DENV serotypes to obtain an approximate EC<sub>90</sub> value (Appendix A). We also tested the cytotoxicity of compounds using an ATP proliferation assay (Niles et al., 2007), allowing us to calculate an LD<sub>90</sub> value. The Gray lab confirmed that all active compounds were not able to inhibit the proliferation of Bcr-Abl transformed Ba/F3 cells (IC<sub>50</sub> >10  $\mu$ M), showing that the compounds are either cell-impermeable or inactive against cellular Abl kinases.

We identified a total of twenty-three disubstituted pyrimidines that inhibited DENV2 infectivity with EC<sub>90</sub> values equal to or lower than that of GNF-2. Testing these compounds against other DENV serotype strains revealed a wide range of activities,

consistent with our hypothesis that E is target of these compounds and mediates their effect on DENV infectivity (Appendix A). The DENV3 strain THD3 was insensitive to many of the compounds, with no calculable EC<sub>90</sub> value for 15 out of the 23 compounds, mirroring its lack of sensitivity to GNF-2 and c-GNF-2. On the other end of the spectrum, the DENV1 strain had a high level of sensitivity to the presence of many compounds, with EC<sub>90</sub> values in the single-digit micromolar against 18 out of 23 compounds, and EC<sub>90</sub> values against the other four compounds were all under 20 µM. The DENV1 results are particularly surprising since DENV2 was the focus of our initial SAR study, and we only identified nine out of 21 compounds that had single-digit micromolar EC<sub>90</sub> values against DENV2. Notably, one compound (CVM-7-128-B) was capable of inhibiting all four serotypes with EC<sub>90</sub> values below 15 µM, suggesting that it may be possible to develop pan-serotype inhibitors.

## **DISCUSSION**

In this chapter, we have identified an unexpected, previously unknown viral target for GNF-2, known to be a small molecule inhibitor of intracellular Abl kinases. We found that GNF-2 reduces DENV infectivity as measured in yield reduction assays when the compound is pre-incubated with virus inoculum. In addition, a combination of biochemical, cellular, and chemical experiments collectively indicate that the target of GNF-2 is the DENV envelope protein in its pre-fusion form on the virion surface. These data do not address how binding of GNF-2 to the DENV E protein leads to a block in viral entry; the potential mechanisms of action of GNF-2 and related anti-DENV

compounds generated in our medicinal chemistry study are the focus of the following chapter.

Over the last decade, there has been an increased focus on small molecule and peptide inhibitors of viral entry as potential antiviral agents. Entry is considered an attractive target for therapeutic intervention since it is an essential step in the viral life cycle and occurs early in the viral life cycle before genome replication, thus minimizing the chance that the virus may be able to actively evolve an escape mutation. In addition, viral entry occurs in distinct, specific steps often involving discrete cellular receptors or pathways, thus making it a more tractable target than some downstream steps that often use a variety of cellular proteins, not all of them known. However, as demonstrated by small molecule HIV entry inhibitors (Araújo et al., 2012; Lu et al., 2012; Westby et al., 2007) as well as work discussed in Chapter 4 of this dissertation, viruses are still able to develop resistance to small molecule or peptide entry inhibitors.

Virus entry is a multistep process, including initial attachment to cells, uptake into cells, and fusion, and there are inhibitors that have been shown to act during all of these steps (Baldick et al., 2010; Chen et al., 1997; Kilby et al., 1998; Schmidt et al., 2012). For DENV, the E protein on the surface of the virion is responsible for mediating all steps of viral entry, and, consistent with our results in the fluorescence polarization assay with GNF-2-FITC, has been shown to bind several small molecule or peptide DENV entry inhibitors (Schmidt et al., 2012; Schmidt et al., 2010a; Zhou et al., 2008) and hypothesized to be the target of several others (Hrobowski et al., 2005; Kampmann et al., 2009; Poh et al., 2009; Wang et al., 2009; Yennamalli et al., 2009). No experimental evidence unequivocally demonstrating the binding site on the E protein binding of GNF-2

or previous DENV entry inhibitors has been reported, although many inhibitors have been hypothesized to bind in the interface between domains I and II of E. This region is a ligand-binding “pocket” that has been shown to contain a single molecule of beta-octoglucoside (BOG) in pre-fusion dimer crystal structures and is hypothesized to be critical for conformational rearrangements of E that occur during its mediation of viral fusion (Modis et al., 2003). Many DENV entry inhibitors were identified via virtual screening against this pocket (Kampmann et al., 2009; Poh et al., 2009; Wang et al., 2009), although other studies have identified entry inhibitors by focusing on other regions of the E protein (Schmidt et al., 2012; Schmidt et al., 2010a; Yennamalli et al., 2009). Thus, the location of binding on E for all DENV entry inhibitors remains an open question.

Consistent with targeting of the DENV2 E protein by GNF-2, we found that GNF-2 and its analogs identified in the SAR study had a wide range of activities against other DENV serotypes. These results echo previously published DENV entry inhibitors that were tested either against other serotypes or flaviviruses. One group showed DENV2-derived peptides (Schmidt et al., 2010b) as well as small molecule entry inhibitors (Schmidt et al., 2012) had a range of activity against other DENV serotypes, but no activity against WNV (Kunjin strain). Another study found that a DENV2 entry inhibitor (IC<sub>50</sub> value 1.2 µM) had activity against WNV (Kunjin strain) as well as YFV (Kampmann et al., 2009). The most broadly tested compound published to date, NITD6, had measurable activity against all DENV serotypes as well as YFV, WNV, and JEV (Wang et al., 2009). However, there were wide variations in potencies; EC<sub>50</sub> values ranged from 68 nM against DENV2 to 1.42 µM against JEV. It is interesting that several studies made note that DENV3 strains were insensitive to both small molecule and

peptide inhibitors of DENV entry; for NITD6, DENV3 had an EC<sub>50</sub> value over four times higher than against DENV1 or DENV2 (Wang et al., 2009). One group (Schmidt et al., 2012; Schmidt et al., 2010b) found that DENV3 was less sensitive than other serotypes to several peptide and small molecule inhibitors, while modeling done by another group (Yennamalli et al., 2009) suggested that the DENV3 envelope protein had lower affinity for their small molecule entry inhibitor than even the WNV envelope was predicted to have. This mirrors our results with GNF-2 and its analogs, in which we observed a one-log drop in DENV3 yield with only seven of the twenty-five total molecules tested. Further exploration of this pattern of DENV3 insensitivity to entry inhibitors could yield information that may help to inform the design of future antivirals targeting the entry step or the E protein.

Although DENV3 was not inhibited by many of our GNF-2 analogs, it was sensitive to some. In particular, compound 7-128-B was able to reduce DENV yield by one log in all four serotypes at concentrations of 15  $\mu$ M or less. This result shows that it is possible to have pan-serotype small molecule inhibitors of DENV entry. Closer examination of the results of our SAR study may provide useful information for the design of future entry inhibitors that use GNF-2 as a scaffold and inhibit all DENV serotypes with greater potency.

GNF-2's anti-DENV activity during entry is not as potent as most other published DENV entry inhibitors, but it is unique among these entry inhibitors due to the fact that it also inhibits DENV at a later stage of the viral life cycle, most likely via its inhibition of cellular Abl kinases. The unexpected observation that GNF-2 acts at two points in the viral life cycle leads to the possible concept of dual-action viral inhibitors. This concept

has been tentatively explored previously in other viruses. One HIV study identified a peptide that inhibits glycoprotein gp120 interactions with both of its cell protein ligands (Gopi et al., 2006), while another study discovered a small molecule that inhibits two separate steps of the HIV integration process (Tsiang et al., 2012). For rhinovirus, an inhibitor was identified that prevented activity of two separate viral proteases (Wang et al., 1998). However, our results for GNF-2 reveal a molecule that inhibits not at two points during one step of the viral life cycle, but rather acts at two separate points in the viral life cycle and is likely doing so via two separate targets. This concept is not without precedent; a dendrimer was identified with the capacity to inhibit both herpes simplex virus (HSV) entry as well as late stages of viral replication (Gong et al., 2002), although potential targets were not explored. We propose that it may be possible to use rational design to identify molecules that can inhibit viruses via two separate targets at separate points in the viral life cycle. The targets could be viral, cellular, or both, as we believe is the case for GNF-2. The challenging part of this approach would be to balance optimization of inhibitor activity between the two targets. While such dual-action inhibitors would be attractive due to their potentially higher barriers to viral resistance, this proposition remains to be experimentally explored.

In summary, this chapter describes the previously unknown ability of GNF-2 to inhibit dengue virus entry in an Abl-independent manner. We used cellular, biochemical, and chemical approaches to characterize this activity and determined that GNF-2 most likely inhibits DENV entry via interactions with the pre-fusion envelope dimer on the virion surface. Our follow-up work in the next two chapters focuses on GNF-2 analogs that were identified through a structure-activity relationship study and explores both

potential mechanism of action of the small molecules as well as the question of virus resistance to the inhibition of entry.



## **METHODS AND MATERIALS**

### **Cells and virus:**

BHK-21 cells were maintained in MEM-alpha containing 5% FBS with 5% CO<sub>2</sub>. C6/36 were maintained in L-15 media with 10% FBS and no gas exchange. All virus stocks were grown in C6/36 cells in L-15 media and 2% FBS, and viral titer was determined by plaque- or focus-forming assay in the appropriate cell line (see below). C6/36 cells were infected for 1 hour with 3 mL of DENV diluted in EBSS, after which time media was added (for a total volume of 20 mL) and the infection proceeded for 4-5 days, until lifting of the cellular monolayer was observed. Supernatant was harvested and spun at 1200 rpm for 5 minutes to remove cell debris, after which glycerol was added (final concentration 20%) and samples were aliquoted and frozen at -80°C.

### **Order of addition experiments:**

Experiments were carried out in BHK-21 cells using DENV2 NGC in the presence of 15 µM of GNF-2 or imatinib at times noted in schematic. All virus was diluted in EBSS to achieve a multiplicity of infection (MOI) of 1. Cells or virus were pre-incubated in 100 µL medium containing compounds for one hour at 37°C. For co-infection, 15 µM of compound was added to the virus inoculum directly before addition to cells. After an initial one hour infection, cells were washed twice with 1X PBS to remove unbound compound and virus and 1 mL cell medium (MEM-alpha with 2% FBS) was added. Medium was replaced with compound-containing medium at the times noted in schematic. Twenty-four hours after initial infection, culture supernatant was collected and viral titer was determined by plaque-forming assay.

**Plaque- and focus-forming assays:**

To determine viral titer,  $10^5$  cells/well (BHK-21 cells for DENV1, 2, and 4; Vero for DENV3) were plated in a 24-well plate and left overnight at 37°C to form a confluent monolayer. Ten-fold serial dilutions of the supernatant were made in Earle's balanced salt solution (EBSS) and 100  $\mu$ L of the dilutions was used to infect each well for one hour at 37°C, after which cells were washed once with 1X PBS. Overlay medium was added (1.05% CMC with MEM $\alpha$ , 2% FBS, Pen/Strep for BHK-21; 1.05% CMC with DMEM, 2% FBS, Pen/Strep for Vero) and plates were incubated for 5 (BHK-21) or 6 days (Vero). Assays were washed twice with 1X PBS to remove overlay medium and cells were fixed. For DENV2, plaque-forming units (pfu/mL) were measured by staining the cells with crystal violet and counting the number of plaques. For DENV1, DENV3, and DENV4, cells were fixed with 3.7% formaldehyde for 15 minutes at room temperature, followed by a 15 minute incubation at RT with 20mM NH<sub>4</sub>Cl. Cold 60/40 (vol/vol) MeOH/Acetone was added and plates were kept at -20°C until staining (30 minutes – overnight). To visualize foci, monolayers were blocked with 1% FBS and 0.1% Tween-20 in 1X PBS for 10 minutes at RT and incubated with 1:1000 dilution of mouse monoclonal antibody 4G2 for 30 minutes at 37°C. Plates were incubated with horseradish peroxidase goat-anti-mouse antibody (1:500) for 30 minutes at 37°C and foci were visualized using the Vector VIP substrate kit (Vector Labs, Cat#SK4600).

**EC<sub>90</sub> values:**

Compound EC<sub>90</sub> values were determined by performing a 6 point titration curve with compounds either added immediately after infection and present for the 24 hours of infection (imatinib and GNF-2), or pre-incubated with the virus inocula for 45 minutes at

37°C and added to cells for a one hour infection (GNF-2 and all analogs). Cells were infected at MOI 1; after 24 hours, supernatants were harvested and PFAs or FFAs were performed to determine viral titer. “EC<sub>90</sub> of entry” describes compounds that were tested by incubating with DENV inocula for 45 minutes at 37°C prior to a one-hour infection of cells before being removed along with unbound virus by washing. Viral titers were graphed, and the EC<sub>90</sub> value was determined to be the point at which a one-log drop in titer was observed compared to a DMSO control. EC<sub>90</sub> values of entry: NITD6 – 200 nM; GNF-2 – 18 µM. EC<sub>90</sub> values for cellular treatment: GNF-2 – 8 µM; imatinib – 8 µM.

#### **Additivity of compounds:**

All compounds were used at EC<sub>90</sub> values that were empirically determined (see previous). Virus inocula were incubated with compounds in 100 µL for 45 minutes at 37°C prior to a one-hour infection of BHK-21 cells. After infection, cells were washed twice with 1X PBS and 1 mL of fresh medium was added. Twenty-four hours later, supernatants were harvested and viral titer was determined by PFA.

#### **Pull down of DENV:**

Dengue virus was purified from infected C6/36 cell supernatant by incubation overnight with PEG8000 to precipitate virus. The virus was pelleted (30 minutes, 9000g), resuspended in TAN buffer, placed over a two-step gradient (40% w/v potassium tartrate with 30% glycerol, 10% w/v potassium tartrate with 7.5% glycerol), and spun for 2.5 hours at 35,000 rpm at 4°C. Gradient fractions containing virions (as determined by Western blot against the E and C protein) were pooled and potassium tartrate was removed by washing with TAN buffer (15 mM TEA, 100 mM NaCl, pH 8.0) and concentrating the virus in an Amicon filter (Millipore, Cat. # UFC810024). Purified

dengue virions (between 300-400 ng, as measured by Bradford assay) were incubated with varying concentrations of GNF-2-biotin for 45 minutes at 37°C in 200 µL HNE buffer. Meanwhile, streptavidin resin (GE Biosciences, Cat. #17-5113-01) was rinsed twice with HNE buffer, blocked with 5% FBS for one hour at 4°C, and resuspended in HNE buffer. Twenty µL of resin was added to the compound and virus and incubated for twenty minutes at 4°C. Each sample was washed ten times with 1 mL HNE buffer before being resuspended in 20 µL Laemmli sample buffer without beta-mercaptoethanol (BioRad, Cat. #161-0737). Samples were boiled (>95°C) for twenty minutes, spun down, and run on a 10% SDS-PAGE gel for separation. Proteins were transferred to PVDF membrane by semi-dry transfer (10V, 1.5 hrs) and membranes were blotted with 4G2 antibody for detection of the DENV E protein. Immunoblots were visualized by chemiluminescence (Pierce ECL reagent cat#32106).

#### **Fluorescence microscopy of DENV and CY5-GNF2:**

Vero cells ( $3 \times 10^4$ ) were plated on coverslips for infection with purified dengue virions (0.5 µg) that had been incubated with 25 µM GNF2-Cy5 at 37°C for 1.5 hours. Cells were infected for 30 minutes at 37°C, then washed three times with 1X PBS to remove any unbound virus or compound and fixed with methanol. Coverslips were stained for the DENV E protein (US Biological, Cat. #D2810-05, 1:200) and visualized using FITC-conjugated goat anti-mouse secondary (Jackson Laboratories Cat. #115-095-146, 1:400). Images are at 600X magnification and typical of a broader field of view.

#### **Fluorescence polarization assay:**

Binding experiments were carried out in Corning, low-volume 384 well microplates and analyzed in a *PerkinElmer EnVision* instrument (excitation wavelength, 485nm; emission

wavelength, 535 nm). Stock concentrations of GNF-2-FITC were made in DMSO and dissolved in assay buffer immediately before use. Increasing concentrations of protein were used with a constant concentration of compound (25 nM). Each well contained a final volume of 20uL in TAN buffer.

## REFERENCES:

- Adrian, F.J., Ding, Q., Sim, T., Velentza, A., Sloan, C., Liu, Y., Zhang, G., Hur, W., Ding, S., Manley, P., *et al.* (2006). Allosteric inhibitors of Bcr-abl-dependent cell proliferation. *Nat Chem Biol* 2, 95-102.
- Araújo, L.A.L., Junqueira, D.M., de Medeiros, R.M., Matte, M.C.C., and Almeida, S.E.d.M. (2012). Naturally occurring resistance mutations to HIV-1 entry inhibitors in subtypes B, C, and CRF31\_BC. *Journal of Clinical Virology* 54, 6-10.
- Baldick, C.J., Wichroski, M.J., Pendri, A., Walsh, A.W., Fang, J., Mazzucco, C.E., Pokornowski, K.A., Rose, R.E., Eggers, B.J., Hsu, M., *et al.* (2010). A Novel Small Molecule Inhibitor of Hepatitis C Virus Entry. *PLoS Pathog* 6, e1001086.
- Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Müller, M., Druker, B.J., and Lydon, N.B. (1996). Inhibition of the Abl Protein-Tyrosine Kinase in Vitro and in Vivo by a 2-Phenylaminopyrimidine Derivative. *Cancer Research* 56, 100-104.
- Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., and Marks, R.M. (1997). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* 3, 866-871.
- Choi, Y., Seeliger, M.A., Panjarian, S.B., Kim, H., Deng, X., Sim, T., Couch, B., Koleske, A.J., Smithgall, T.E., and Gray, N.S. (2009). N-myristoylated c-Abl tyrosine kinase localizes to the endoplasmic reticulum upon binding to an allosteric inhibitor. *J Biol Chem* 284, 29005-29014.
- Chu, J.J.H., and Yang, P.L. (2007). c-Src protein kinase inhibitors block assembly and maturation of dengue virus. *Proceedings of the National Academy of Sciences* 104, 3520-3525.
- Gong, Y., Matthews, B., Cheung, D., Tam, T., Gadawski, I., Leung, D., Holan, G., Raff, J., and Sacks, S. (2002). Evidence of dual sites of action of dendrimers: SPL-2999 inhibits both virus entry and late stages of herpes simplex virus replication. *Antiviral Research* 55, 319-329.
- Gopi, H.N., Tirupula, K.C., Baxter, S., Ajith, S., and Chaiken, I.M. (2006). Click Chemistry on Azidoproline: High-Affinity Dual Antagonist for HIV-1 Envelope Glycoprotein gp120. *ChemMedChem* 1, 54-57.
- Hennequin, L.F., Allen, J., Breed, J., Curwen, J., Fennell, M., Green, T.P., Lambert-van der Brempt, C., Morgentin, R., Norman, R.A., Olivier, A., *et al.* (2006). N-(5-Chloro-1,3-benzodioxol-4-yl)-7-[2-(4-methylpiperazin-1-yl)ethoxy]-5- (tetrahydro-2H-pyran-4-yloxy)quinazolin-4-amine, a Novel, Highly Selective, Orally Available, Dual-Specific c-Src/Abl Kinase Inhibitor†. *Journal of Medicinal Chemistry* 49, 6465-6488.

- Holen, I., Str, P.E., Gordon, P.B., Fengsrud, M., Berg, T.O., and Seglen, P.O. (1995). Inhibition of Autophagy and Multiple Steps in Asialoglycoprotein Endocytosis by Inhibitors of Tyrosine Protein Kinases (Tyrophostins). *Journal of Biological Chemistry* 270, 12823-12831.
- Hrobowski, Y.M., Garry, R.F., and Michael, S.F. (2005). Peptide inhibitors of dengue virus and West Nile virus infectivity. *Virology Journal* 2.
- Kampmann, T., Yennamalli, R., Campbell, P., Stoermer, M.J., Fairlie, D.P., Kobe, B., and Young, P.R. (2009). In silico screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses. *Antiviral Research* 84, 234-241.
- Kilby, J.M., Hopkins, S., Venetta, T.M., DiMassimo, B., Cloud, G.A., Lee, J.Y., Alldredge, L., Hunter, E., Lambert, D., Bolognesi, D., *et al.* (1998). Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nature Medicine* 4, 6.
- Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., *et al.* (2002). Structure of Dengue Virus: Implications for Flavivirus Organization, Maturation, and Fusion. *Cell* 108, 717-725.
- Lambeth, C.R., White, L.J., Johnston, R.E., and de Silva, A.M. (2005). Flow Cytometry-Based Assay for Titrating Dengue Virus. *Journal of Clinical Microbiology* 43, 3267-3272.
- Lindenbach, B.D., and Rice, C. (2001). Flaviviridae: The Viruses and Their Replication. *Fundamentals of Virology*, eds Knipe and Howley 1, 589-639.
- Lu, L., Tong, P., Yu, X., Pan, C., Zou, P., Chen, Y.-H., and Jiang, S. (2012). HIV-1 variants with a single-point mutation in the gp41 pocket region exhibiting different susceptibility to HIV fusion inhibitors with pocket- or membrane-binding domain. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1818, 2950-2957.
- Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America* 100, 6986-6991.
- Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2005). Variable Surface Epitopes in the Crystal Structure of Dengue Virus Type 3 Envelope Glycoprotein. *Journal of Virology* 79, 1223-1231.
- Niles, A.L., Moravec, R.A., Eric Hesselberth, P., Scurria, M.A., Daily, W.J., and Riss, T.L. (2007). A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers. *Analytical Biochemistry* 366, 197-206.

- Okram, B., Nagle, A., Adrián, F.J., Lee, C., Ren, P., Wang, X., Sim, T., Xie, Y., Wang, X., Xia, G., *et al.* (2006). A General Strategy for Creating “Inactive-Conformation” Abl Inhibitors. *Chemistry & Biology* 13, 779-786.
- Poh, M.K., Yip, A., Zhang, S., Priestle, J.P., Ma, N.L., Smit, J.M., Wilschut, J., Shi, P.-Y., Wenk, M.R., and Schul, W. (2009). A small molecule fusion inhibitor of dengue virus. *Antiviral Research* 84, 260-266.
- Schmidt, A.G., Lee, K., Yang, P.L., and Harrison, S.C. (2012). Small-Molecule Inhibitors of Dengue-Virus Entry. *PLoS Pathog* 8, e1002627.
- Schmidt, A.G., Yang, P.L., and Harrison, S.C. (2010a). Peptide Inhibitors of Dengue-Virus Entry Target a Late-Stage Fusion Intermediate. *PLoS Pathog* 6, e1000851.
- Schmidt, A.G., Yang, P.L., and Harrison, S.C. (2010b). Peptide Inhibitors of Flavivirus Entry Derived from the E Protein Stem. *Journal of Virology* 84, 6.
- Shah, N.P., Tran, C., Lee, F.Y., Chen, P., Norris, D., and Sawyers, C.L. (2004). Overriding Imatinib Resistance with a Novel ABL Kinase Inhibitor. *Science* 305, 399-401.
- Tsiang, M., Jones, G.S., Niedziela-Majka, A., Kan, E., Lansdon, E.B., Huang, W., Hung, M., Samuel, D., Novikov, N., Xu, Y., *et al.* (2012). New Class of HIV-1 Integrase (IN) Inhibitors with a Dual Mode of Action. *Journal of Biological Chemistry* 287, 21189-21203.
- van der Schaar, H.M., Rust, M.J., Waarts, B.-L., van der Ende-Metselaar, H., Kuhn, R.J., Wilschut, J., Zhuang, X., and Smit, J.M. (2007). Characterization of the Early Events in Dengue Virus Cell Entry by Biochemical Assays and Single-Virus Tracking. *Journal of Virology* 81, 12019-12028.
- Wang, Q.-Y., Patel, S.J., Vangrevelinghe, E., Xu, H.Y., Rao, R., Jaber, D., Schul, W., Gu, F., Heudi, O., Ma, N.L., *et al.* (2009). A Small Molecule Dengue Virus Entry Inhibitor. *Antimicrob Agents Chemother*, AAC.01148-01108.
- Wang, Q.M., Johnson, R.B., Jungheim, L.N., Cohen, J.D., and Villarreal, E.C. (1998). Dual Inhibition of Human Rhinovirus 2A and 3C Proteases by Homophthalimides. *Antimicrobial Agents and Chemotherapy* 42, 916-920.
- Westby, M., Smith-Burchnell, C., Mori, J., Lewis, M., Mosley, M., Stockdale, M., Dorr, P., Ciaramella, G., and Perros, M. (2007). Reduced Maximal Inhibition in Phenotypic Susceptibility Assays Indicates that Viral Strains Resistant to the CCR5 Antagonist Maraviroc Utilize Inhibitor-Bound Receptor for Entry. *Journal of Virology* 81, 2359-2371.
- WHO (2012). Dengue and severe dengue.



Yennamalli, R., Subbarao, N., Kampmann, T., McGeary, R., Young, P., and Kobe, B. (2009). Identification of novel target sites and an inhibitor of the dengue virus E protein. *Journal of Computer-Aided Molecular Design*.

Zhang, J., Adrian, F.J., Jahnke, W., Cowan-Jacob, S.W., Li, A.G., Iacob, R.E., Sim, T., Powers, J., Dierks, C., Sun, F., *et al.* (2010). Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature* **463**, 501-506.

Zhou, Z., Khaliq, M., Suk, J.-E., Patkar, C., Li, L., Kuhn, R.J., and Post, C.B. (2008). Antiviral Compounds Discovered by Virtual Screening of Small Molecule Libraries against Dengue Virus E Protein. *ACS Chemical Biology* **3**, 765-775.

**Chapter 3:** Exploring the mechanism of action of select GNF-2 analogs during dengue virus entry

**Acknowledgments:**

I would like to thank Dr. Aaron Schmidt in Dr. Stephen Harrison's lab for helpful discussions and teaching me several of the *in vitro* assays featured in this chapter. I would also like to express my appreciation to Drs. Chandra Miduturu and Jinhua Wang in the Gray lab who synthesized all the compounds used in this chapter and kept me steadily supplied. Thanks to members of the Yang lab, past and present, for helpful discussions and suggestions along the way.

## SUMMARY

In earlier work, we discovered that GNF-2, a small molecule inhibitor of cellular Abl kinases, inhibited dengue virus (DENV) infectivity via interactions with the envelope protein on the virion surface. We performed a focused structure-activity relationship study and identified twenty-three small molecule analogs of GNF-2 that had no cellular activity against Abl kinases yet inhibited DENV infectivity more potently than GNF-2. We chose two of the most potent compounds, 2-12-2 and 8-24-3 (DENV2 EC<sub>90</sub> values during entry ~5  $\mu$ M), for further characterization and use in mechanism of action studies. We found that the inhibition by these compounds was temperature-dependent, could be overcome with higher initial MOIs, and may be reversible. In mechanistic studies we found that neither 2-12-2 nor 8-24-3 inhibited attachment of DENV to cells, the first step in viral entry. When we measured DENV fusion *in vitro*, we found that, after treatment with the compounds, DENV did not complete the fusion process, although virions still stably associated with target membranes in the presence of the compounds. We hypothesize that our compounds inhibit DENV entry by interacting with the E protein on the surface of the virion in a way that prevents viral fusion with the cellular membrane inside endosomes, thus blocking release of the viral genome into the cytoplasm for replication.

## INTRODUCTION

Dengue virus (DENV) is a mosquito-borne virus that can cause severe febrile disease in humans. Over forty percent of the world's population is at risk to contract this virus, and there are currently no specific treatments or vaccines available (WHO, 2012). Dengue is a small, enveloped virion that in its mature, infectious form has one major surface protein, the envelope (E) protein, which mediates all steps of viral entry, including attachment to cells and fusion between the viral and cellular membranes inside endosomes (Harrison, 2005). There are 180 copies of the E protein on the surface of the virus, which on the mature virion form 90 homodimers arranged in a tight herringbone pattern (Kuhn et al., 2002). E consists of three distinct domains as well as a stem region and transmembrane domain; domain I has a  $\beta$ -barrel shape and is centrally located, domain II is finger-like in structure and contains the fusion loop at the end distal to the viral membrane, and domain III contains the purported receptor binding domain (Mukhopadhyay et al., 2005). The stem region consists of two alpha helices that, on the mature virion surface, sit underneath the rest of the E protein, close to the viral membrane.

E undergoes a series of conformational changes as it mediates fusion between the viral and cellular membranes (Figure 1-3). The pre- and post-fusion structures of E have been solved, allowing deduction of the conformational rearrangements that likely occur during fusion (Modis et al., 2003, 2004). The initial trigger for these rearrangements is the low pH of the endosome, which is thought to initially cause dissociation of the E homodimer, allowing extension of domain II and insertion of the fusion loop into the cellular membrane. It is thought that domain III then folds back, moving away from

domain II, which forms new contacts with the stem region as the helices move away from the viral membrane. Collectively, these structural rearrangements bring the viral and cellular membranes in proximity, thereby catalyzing mixing of the outer lipid layers of virus and cell to yield a hemifusion intermediate, followed by mixing of the inner lipid layer and creation of a fusion pore, allowing release of the viral genome into the cellular cytoplasm.

In earlier work, we discovered that GNF-2, a molecule inhibitor of cellular Abl kinases, inhibited DENV infectivity and bound to both purified dengue virions as well as recombinant E protein in its pre-fusion, dimer conformation (Chapter 2). We hypothesized that GNF-2 inhibits DENV infectivity by interacting with the E protein on the virion surface and inhibiting E's functions in viral entry. As part of this work, we conducted a structure-activity relationship study to identify analogs of GNF-2 with increased inhibition of DENV infectivity. We identified twenty-three small molecules that inhibited DENV2 during entry more potently than GNF-2 yet had no measurable inhibition when tested against intracellular Abl kinases (Appendix A).

This chapter describes efforts to learn more about how select GNF-2 analogs inhibit DENV infectivity. To this end, we chose two of the most potent compounds, 2-12-2 and 8-24-3, from the SAR study ( $EC_{90}$  values  $\sim 5 \mu M$ ) for use in cellular and biochemical assays. We examined several parameters, including temperature and time of pre-incubation with virus inocula, to gain a better understanding of how the compounds interact with dengue virions. In addition, to determine the mechanism by which compounds inhibit entry, we examined early and late steps of DENV entry in the presence of compounds.

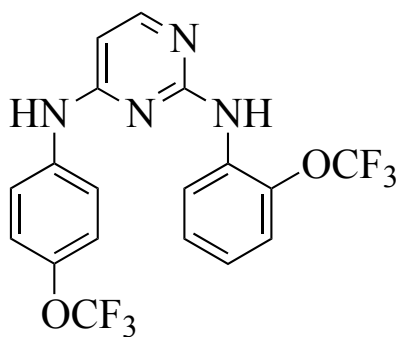
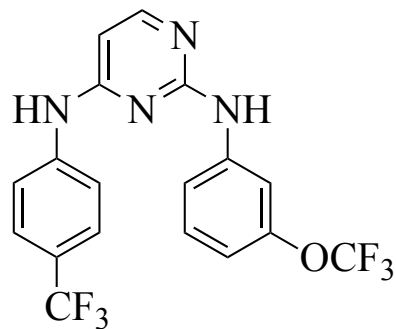
Compounds 2-12-2 and 8-24-3 did not significantly decrease DENV attachment to cells, suggesting that they act at a later step of the viral entry process. Using *in vitro* assays, we found that both compounds inhibit a late step in the viral fusion cascade. We hypothesize that these compounds interact with the DENV E protein and inhibit DENV infectivity by preventing conformational rearrangements necessary for the E protein to mediate pore formation, thus preventing release of the genome into the cytoplasm for replication.

## RESULTS

### Confirmation of GNF-2 analogs as DENV entry inhibitors

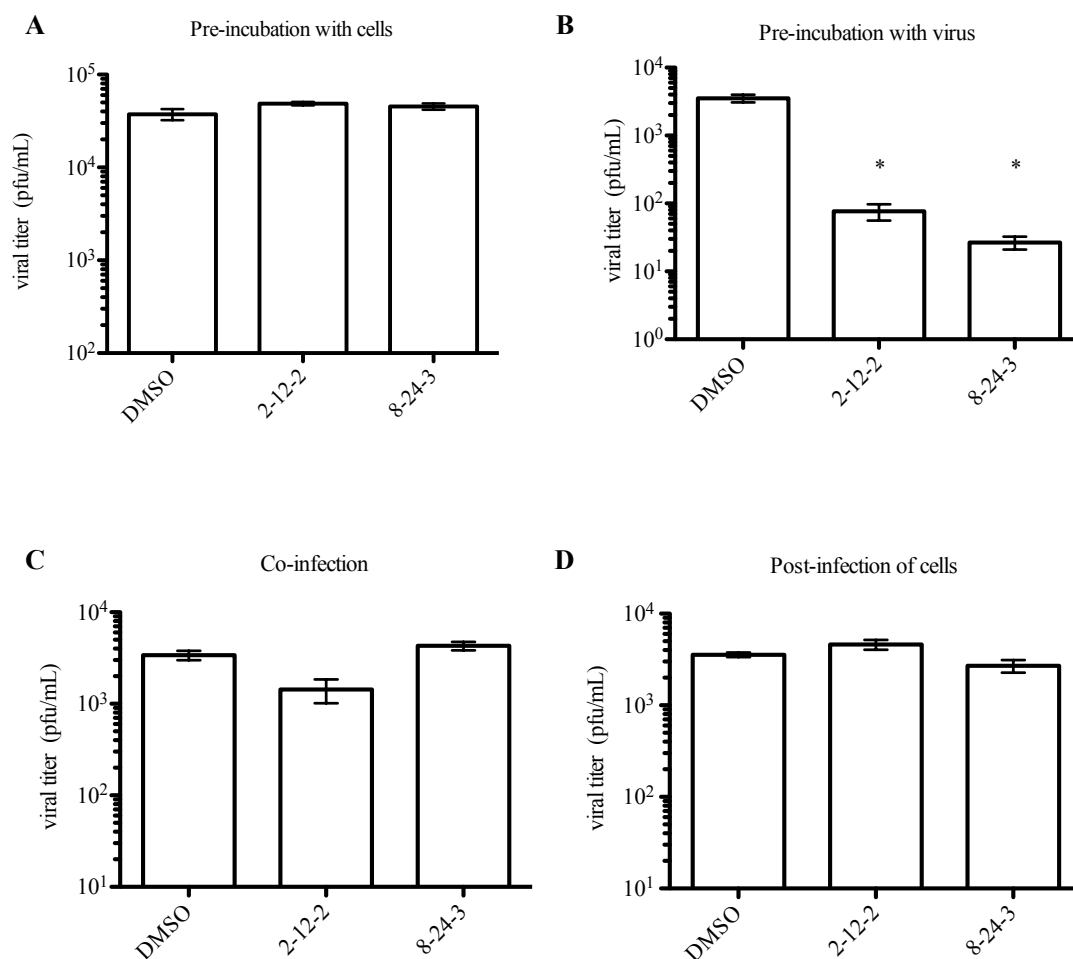
Our structure-activity relationship (SAR) study using GNF-2 as a scaffold identified twenty-three small molecules that significantly lowered DENV2 infectivity at concentrations  $< 25 \mu\text{M}$  (Appendix A). All compounds were individually tested for inhibition of Ba/F3 cell proliferation as a measure of Abl kinase activity and found to be inactive ( $\text{IC}_{50} > 10 \mu\text{M}$ ). We selected two compounds, 2-12-2 and 8-24-3 for further experiments to characterize inhibition of DENV infectivity (Figure 3-1). Compound 2-12-2 had an  $\text{EC}_{90}$  value when present during DENV2 entry of  $\sim 5 \mu\text{M}$  and was one of only two compounds to inhibit DENV3 yield with a single-digit  $\text{EC}_{90}$  value ( $\sim 5 \mu\text{M}$ ), although it was not potent against DENV4 ( $\text{EC}_{90}$  value  $\sim 40 \mu\text{M}$ ). Compound 8-24-3 was the most potent compound against all serotypes other than DENV3 ( $\text{EC}_{90}$  values: DENV1,  $3 \mu\text{M}$ ; DENV2,  $5 \mu\text{M}$ ; DENV4,  $10 \mu\text{M}$ ).

In order to confirm that 2-12-2 and 8-24-3 exerted their anti-DENV activity during viral entry, we performed order of addition experiments (Figure 3-2). As expected,

**A****2-12-2****B****8-24-3**

**Figure 3-1. Structures of small molecules 2-12-2 and 8-24-3** Small molecules were identified in the structure-activity relationship study using GNF-2 as a scaffold. EC<sub>90</sub> values during DENV entry: (A) 2-12-2 – DENV1 4 μM, DENV2 5 μM, DENV3 5 μM, DENV4 40 μM; (B) 8-24-3 – DENV1 3 μM, DENV2 5 μM, DENV3 >40 μM, DENV4 10 μM





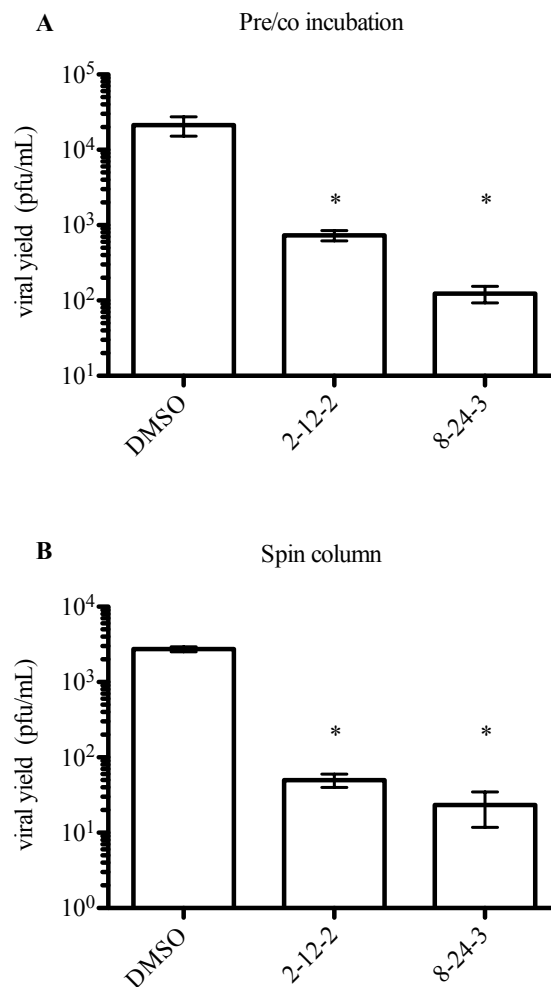
**Figure 3-2. Small molecules lower DENV titer when pre-incubated with virus.**

(A) Cells or (B) virus inocula were pre-incubated with DMSO or 5  $\mu$ M of compounds for 45 minutes at 37°C prior to one-hour initial infection (MOI 1). (C) Compounds were added to cells at the same time as virus inocula for a one hour infection. (D) Compounds were added for one hour after initial infection. Supernatants were harvested 24 hours post-infection and DENV yields were determined by plaque-forming assay. Each bar represents the mean of three replicates with error bars showing standard deviation.

\* indicates p value < 0.01

the compounds did not significantly inhibit DENV when added to cells before, during, or after viral infection. A significant drop in DENV yield was observed when compounds were pre-incubated with virus inocula, consistent with a viral target. In order to ensure that the compounds did not target the cell during the initial hour infection, we also performed pre-incubation of the virus with compounds followed by removal of unbound compound via gel filtration column. Using this pre-incubated virus inocula to infect cells, we observed that DENV infectivity was still significantly reduced, as evidenced by a large decrease in the yield of infectious virus produced by 24 hours post-infection; moreover, this inhibition was of similar magnitude to the inhibition observed when compound was pre-incubated with virus inoculum and then present during the initial infection (Figure 3-3).

As another way to show that the compounds act via a viral target, we performed an MOI titration with the compounds. If the target of our compounds is the dengue virion, as we hypothesized, we would expect that increasing the amount of virus in the initial inocula would overcome the inhibitory effects of the compounds. The EC<sub>90</sub> value of 5  $\mu$ M for 2-12-2 and 8-24-3 had been calculated when cells were infected at a multiplicity of infection (MOI) of 1, so we retained this compound concentration while varying the initial MOI, as measured by plaque-forming units (pfu). Virus and compound were pre-incubated for 45 minutes at 37°C and this mixture was added to cells for one hour. Cells were washed to remove unbound virus and compound, then medium was added and supernatant was harvested 24 hours later to determine DENV titer. We found that doubling the amount of infectious virus in the inoculum (MOI of 2) led to a decrease in inhibition of DENV infectivity by 2-12-2 and 8-24-3, while by an MOI of 4, no



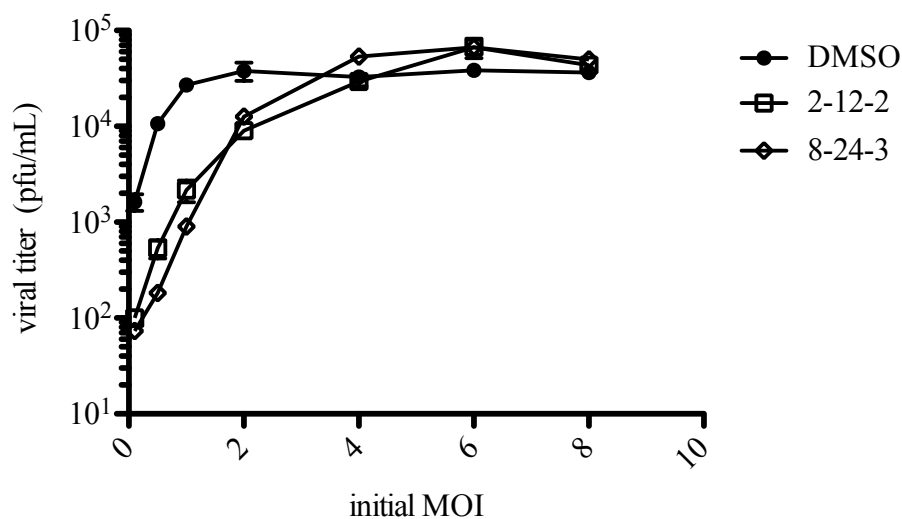
**Figure 3-3. DENV titer is reduced when free compound is removed prior to infection.** (A) DENV was pre-incubated with compound for 45 minutes at 37°C (MOI 1), and mixture was added to cells for the initial one-hour infection. (B) DENV was pre-incubated with compound for 45 minutes at 37°C, and mixture was placed over a Sephadex G-50 spin column to remove unbound compound. Flow through was used to infect cells for one hour. Supernatants were harvested at 24 hours post-infection, and viral yield was determined by plaque-forming assay. Each bar represents the mean of three replicates with error bars showing standard deviation. \* indicates p value < 0.05

difference in DENV yield could be detected between control and compound treated virus (Figure 3-4). This result is consistent with our hypothesis that the compounds act via a viral target.

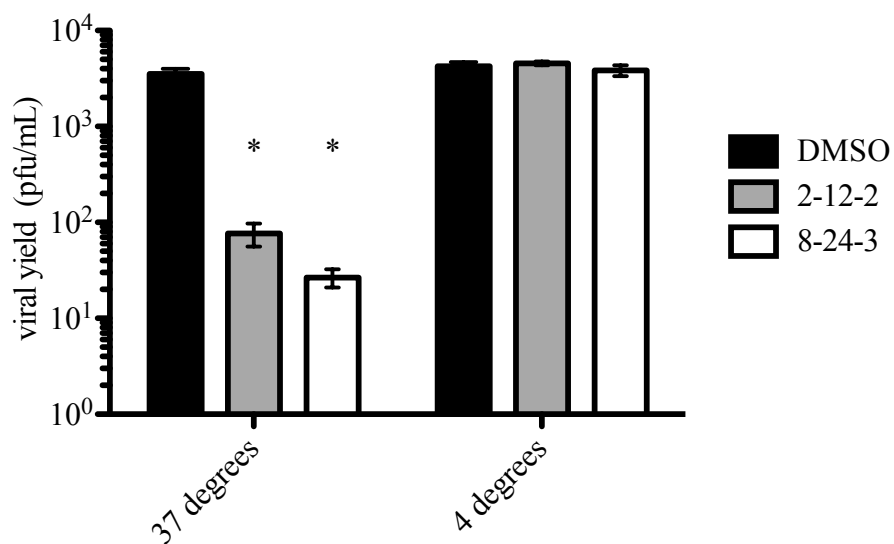
### **Investigating the characteristics of inhibition of DENV entry by compounds**

After confirming that 2-12-2 and 8-24-3 inhibit DENV when pre-incubated with virus inocula and appear to act via a viral target, we wanted to further investigate how the compounds inhibited DENV infectivity. We asked if the anti-DENV activity of 2-12-2 and 8-24-3 was retained when pre-incubation with virus was not done at 37°C. Instead, we pre-incubated compound and virus at 4°C, then added the mixture to cells for an initial one hour infection at 37°C. We observed no inhibition of DENV infectivity with compound treatment under these conditions (Figure 3-5), showing that pre-incubation at 37°C is necessary for the compounds to exert their anti-DENV activity. We hypothesize that thermal motion of the dengue envelope is important in allowing the compounds to bind, possibly through transient exposure of binding site(s).

In all experiments conducted up to this point, compounds 2-12-2 and 8-24-3 had been pre-incubated with virus inocula for 45 minutes. Our previous results showed that addition of compounds and virus to cells simultaneously did not significantly affect DENV infectivity (Figure 3-2), suggesting that some period of pre-incubation is necessary for the compound's anti-DENV activity. We performed a pre-incubation time course with 8-24-3 and 2-12-2 in which cells were infected with compound-treated DENV after increasing pre-incubation times at 37°C. Unbound compound and virus were removed by washing after one hour on cells, and the infection was allowed to proceed for



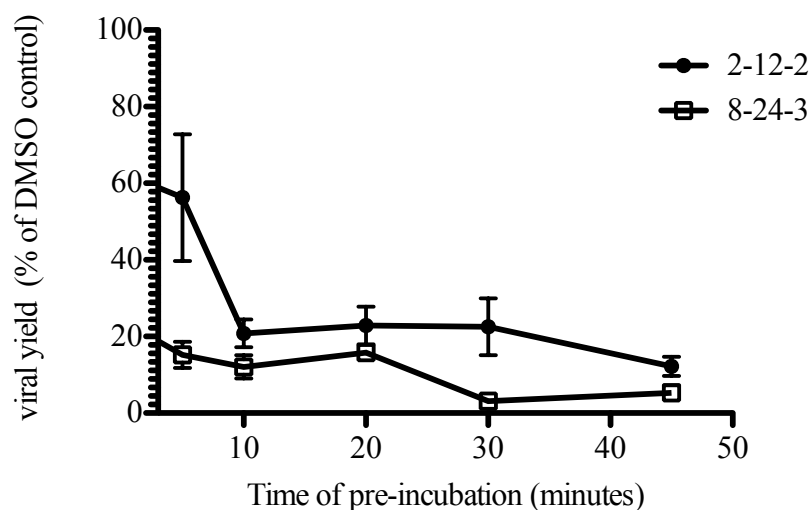
**Figure 3-4. Inhibition by compounds is lost with increasing initial MOI.** Virus inocula (various multiplicities of infection) were pre-incubated with 5  $\mu$ M 2-12-2 or 8-24-3 ( $EC_{90}$  values for MOI 1) for 45 minutes at 37°C, and mixture was added to cells for the initial one-hour infection. Supernatants were harvested at 24 hours post-infection, and viral yield was determined by plaque-forming assay. Each point represents the mean of three replicates with error bars showing standard deviation.



**Figure 3-5. Pre-incubation at 4°C causes loss of inhibition by compounds. DENV2** (MOI 1) was pre-incubated with 5  $\mu$ M of compound at either 37°C (*left*) or 4°C (*right*) for 45 minutes before mixture was added to cells for an initial one hour infection. Supernatants were harvested at 24 hours post-infection, and viral yield was determined by plaque-forming assay. Each bar represents the mean of three replicates with error bars showing standard deviation. \* indicates p value < 0.01

24 hours, at which point supernatants were harvested and DENV titer was measured. With 5  $\mu$ M 8-24-3, we observed an 80% reduction in DENV infectivity compared to a DMSO control after only five minutes pre-incubation, although ten minutes or longer was necessary to observe the full one-log drop in DENV titer observed previously with a 45 minute pre-incubation (Figure 3-6). The anti-DENV activity of 2-12-2 did not occur as quickly; a five minute pre-incubation only led to a 45% reduction compared to the DMSO control, although this dropped to 80% by 10 minutes, and 45 minutes pre-incubation was necessary to achieve the full one-log drop in DENV titer. These results suggest that binding by 2-12-2 and 8-24-3 to virus may not be instantaneous, but a ten minute exposure to virus prior to cellular infection is sufficient to observe a significant decrease in DENV infectivity.

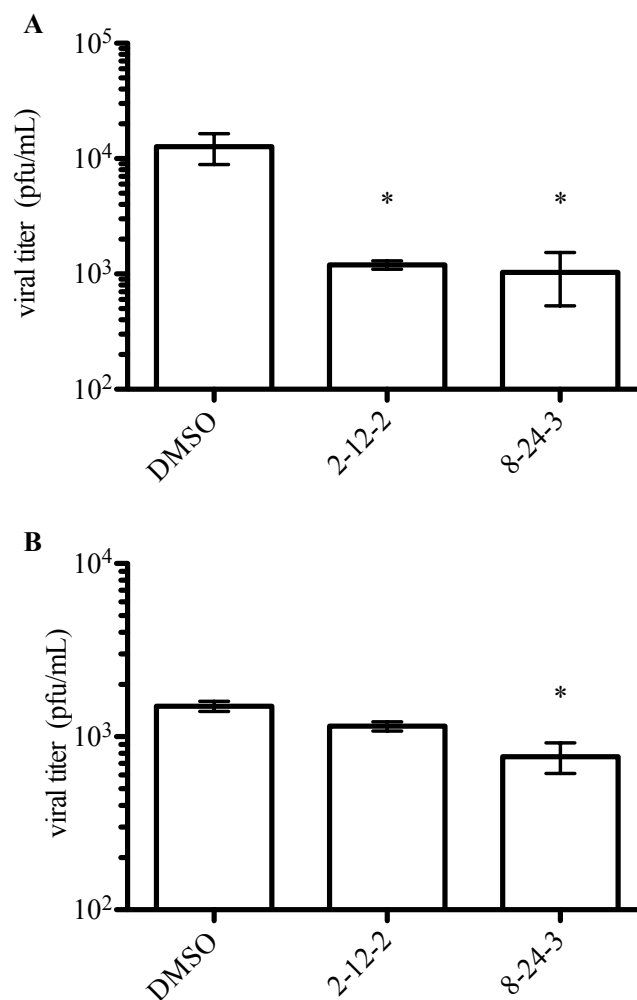
After learning that our compounds significantly inhibited DENV infectivity within ten minutes of pre-incubation, we next asked if we could observe reversion of inhibition by 2-12-2 or 8-24-3 when compounds are diluted below active concentrations. To test this, we pre-incubated DENV with 20  $\mu$ M of the compounds in 5  $\mu$ L for 40 minutes at 37°C, then diluted the mixture 100-fold to bring compound concentrations to 200 nM, well below the value at which we can detect anti-viral activity. We used this diluted mixture either to immediately infect cells or returned the mixture to 37°C for an additional 40 minutes (final MOI 0.4). We found that pre-treatment with 2-12-2 or 8-24-3 significantly inhibited DENV infectivity when mixture was added to cells immediately after the dilution (Figure 3-7). After an additional 40 minute incubation at a higher volume, we found no inhibition of DENV infectivity in 2-12-2 treated virus compared to our DMSO control, suggesting that the virus inocula recovered from treatment with



**Figure 3-6. Pre-incubation time with DENV2 affects magnitude of inhibition.**

DENV2 (MOI 1) was incubated with DMSO or 5  $\mu$ M of compound at 37°C for various lengths of time before addition to cells for a one-hour initial infection. Supernatants were harvested at 24 hours post-infection, and viral yield was determined by plaque-forming assay. Values are expressed as percent of the DMSO control for each time point. Each point represents the mean of three replicates with error bars showing standard deviation.





**Figure 3-7. Inhibition by compounds may be reversible.** Five  $\mu$ L DENV was incubated with 20  $\mu$ M compounds for 45 minutes at 37°C, after which it was diluted 100X fold and either used (*A*) to immediately infect cells or (*B*) incubated an additional 45 minutes at 37°C before being added to cells (final MOI 0.4). Supernatants were harvested 24 hours post-infection, and yield was determined by PFA. Each bar represents the mean of three replicates with error bars showing standard deviation. \* indicates p value < 0.05

2-12-2. Interestingly, we observed that 8-24-3 treated virus was still significantly inhibited relative to the DMSO control even when incubated for 40 minutes at the increased volume (Figure 3-7B), although some reversal of inhibition was clearly observed when compared to the sample infected with 8-24-3 treated virus immediately following dilution (Figure 3-7A). These data, along with the results from the pre-incubation time course, suggest that the inhibition of DENV infectivity with 2-12-2 and 8-24-3 is not due to nonspecific inactivation of DENV. The results with 8-24-3 may be due to a slower off-rate than that of 2-12-2 from the virus, and a longer post-dilution incubation may allow full recovery of DENV infectivity.

### **Compounds do not block DENV attachment to cells**

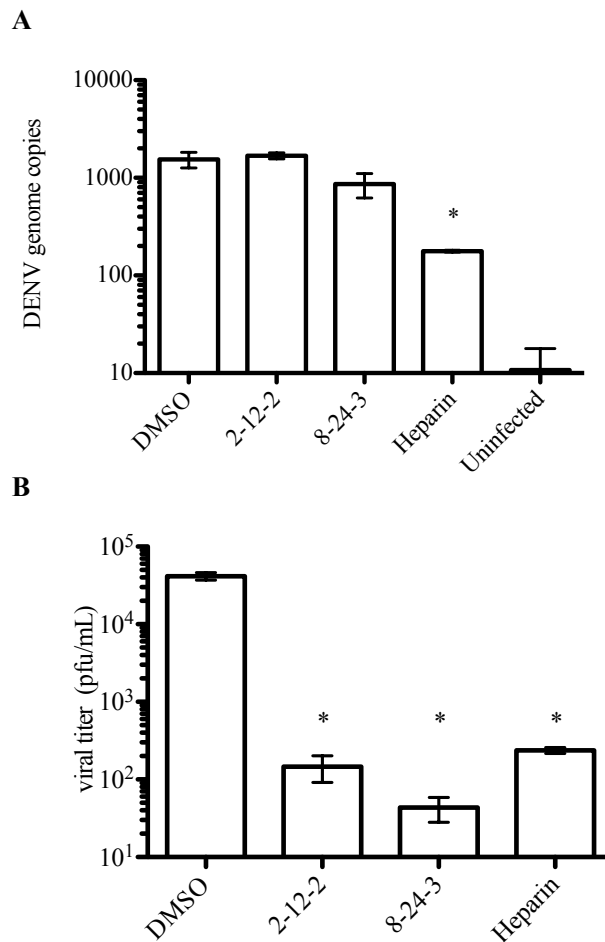
Since DENV entry is a multi-step process, we wanted to explore the mechanism by which 2-12-2 and 8-24-3 exert their anti-DENV activity. We examined whether the first step of viral entry, DENV attachment to cells, was altered in the presence of 2-12-2 or 8-24-3. Previously, it has been shown that at 4°C, DENV attaches to but is not taken up by cells (van der Schaar et al., 2007). Thus, after incubating DENV inocula with compounds at 37°C, all subsequent steps were performed at 4°C and washed with chilled buffer to prevent cellular uptake of virions. DENV (MOI 1) was incubated for 45 minutes at 37°C, moved to 4°C briefly to cool, and then added to chilled cells and rocked gently for 2 hours. Cells were washed multiple times to remove unbound compound and virus, and either total RNA was extracted from cells immediately or infection was allowed to proceed for 24 hours and supernatants were collected. The number of DENV genomes bound was determined by qRT-PCR, and we found that treating virus inocula with 2-12-2

or 8-24-3 did not significantly lower the number of viral genomes bound to cells (Figure 3-8A). When the infection was allowed to proceed, DENV titer was still significantly reduced in the compound-treated conditions compared to the DMSO control (Figure 3-8B). As a positive control, heparin was added to virus inoculum; previous studies show that this inhibits DENV entry by preventing binding of virions to cells (Chen et al., 1997). Taken together, these data show that 2-12-2 and 8-24-3 do not affect attachment of DENV to cells and the inhibition by these compounds occurs downstream of initial DENV attachment to cells.

### **Compounds inhibit a late step of DENV fusion *in vitro***

We next measured the effect of 2-12-2 and 8-24-3 on the last step of DENV entry, the fusion of viral and cellular membranes. DENV fusion with the cellular membrane occurs inside endosomes and is hypothesized to be a multi-step process (Figure 1-3), making it challenging to dissect specific steps of this biochemical pathway in live cells. In order to examine individual steps of DENV fusion, we performed *in vitro* fusion assays in which liposomes are used as a model membrane for fusion with DENV.

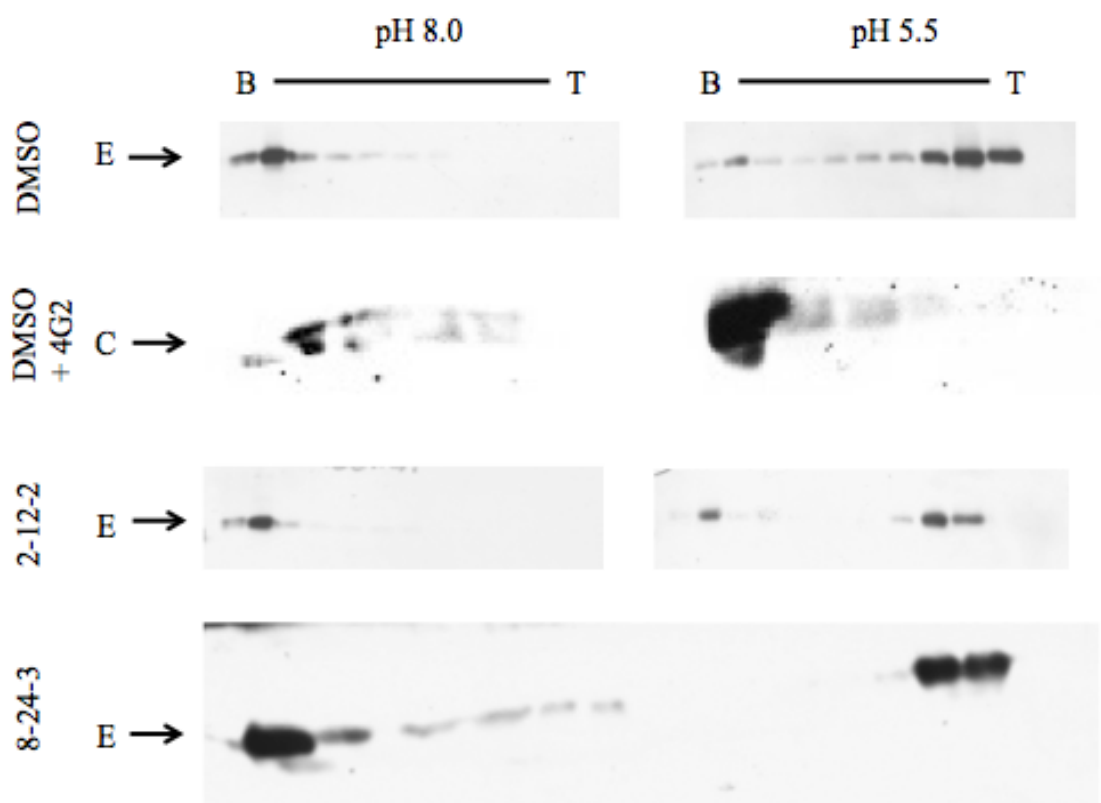
Initially, we examined whether dengue virions formed stable associations with target membranes after incubation with 2-12-2 or 8-24-3. This association is a surrogate for the fusion loop insertion step in viral fusion (Figure 1-3B) and can be detected in a co-floitation assay by determining in which fraction of a sucrose gradient DENV equilibrates. At pH 8.0, DENV fusion loops are not exposed and thus virions will not form stable association with liposomes; DENV equilibrates near the bottom of the gradient. When exposed to pH 5.5, however, fusion loops are exposed and inserted into



**Figure 3-8. Compounds do not block DENV2 attachment to cells.** DENV2 was pre-incubated with DMSO or small molecules at 37°C for 45 minutes before addition to chilled cells. Cells were rocked continuously for one hour at 4°C before being washed to remove any unbound virus. (A) The number of DENV2 genomes bound to cells immediately post-washing, as determined by collecting total RNA and performing qRT-PCR with DENV2 primers. (B) Infection was allowed to proceed for 24 hours, and DENV yield was determined by plaque-forming assay. Each bar represents the mean of three replicates with error bars showing standard deviation. \* indicates p value < 0.01

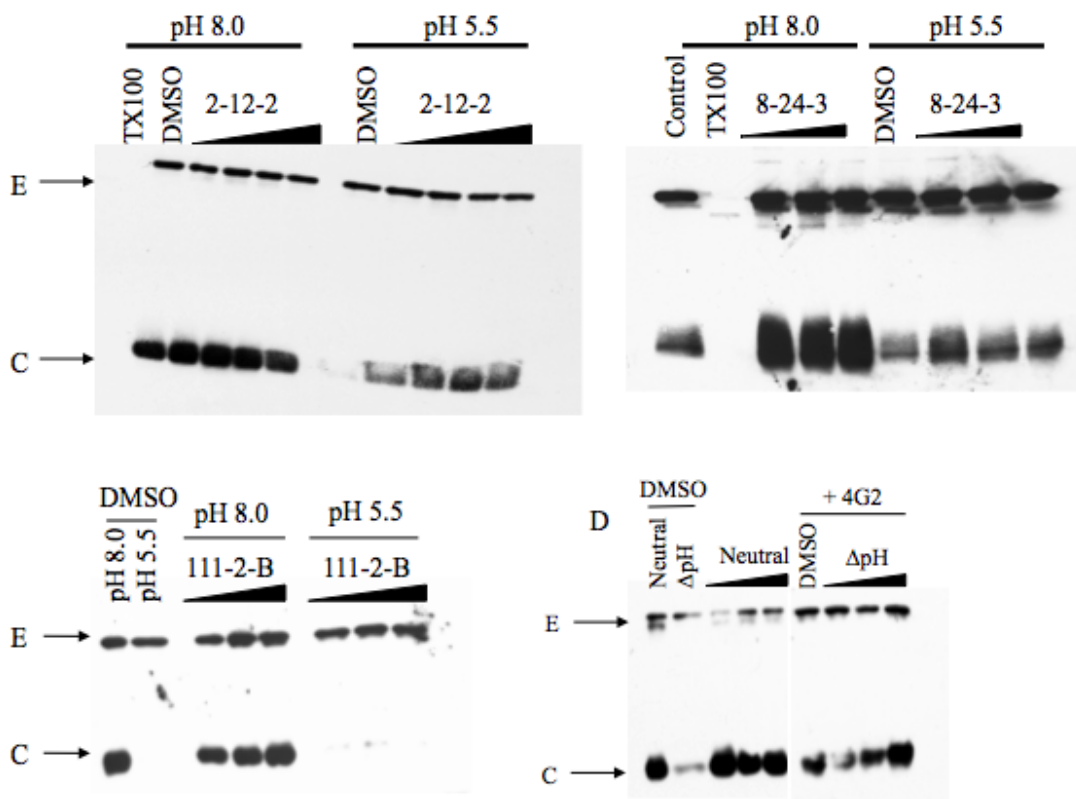
target membranes, so DENV equilibrates at a lower density, near the top of the gradient. Dengue virions are detected by fractionation of the sucrose gradient, precipitation of proteins, and Western blot analysis for the DENV E protein. After control treatment, purified DENV equilibrates near the bottom of the gradient at pH 8.0, while at pH 5.5, the banding pattern shows a shift in DENV sedimentation (Figure 3-9). Treatment of virus with 4G2, an antibody that targets the fusion loops of DENV, caused DENV to equilibrate near the bottom of the gradient even at pH 5.5, confirming that DENV association with liposomes is mediated by the viral fusion loops. Treatment of DENV with 2-12-2 or 8-24-3 revealed the same banding pattern of DENV E in sucrose gradients as the DMSO-treated virus, showing that compound treatment of dengue virions does not inhibit stable association of virus with target membranes.

Fusion loop exposure and insertion are early steps in DENV fusion, so we next asked if DENV completes fusion in the presence of 2-12-2 and 8-24-3. Formation of a fusion pore, the last step in DENV fusion (Figure 1-3E), can be measured via a capsid-protection assay (Poh et al., 2009; Schmidt et al., 2010). Successful formation of the fusion pore between dengue virions and trypsin-loaded liposomes permits trypsin access to the contents of the virion, resulting in the degradation of the core (C) protein, an event that can be monitored by immunoblotting. The capsid signal is maintained when virus and liposomes are incubated at pH 8.0, whereas exposure to pH 5.5 causes a loss of capsid signal due to fusion of the virion with the liposome and subsequent digestion of the nucleocapsid by trypsin. DENV2 treated with 2-12-2 and 8-24-3 showed retention of capsid signal under fusion-promoting conditions (Figure 3-10A and B), while treatment of DENV with an inactive control compound, 111-2-B, showed a complete loss of capsid



**Figure 3-9. Compounds do not inhibit DENV association with target membranes.**

Western blot of coflotation assays with purified DENV2 and liposomes. T=top of gradient (lowest density); B=bottom of gradient (highest density) (*Top row*) At pH 8.0, DMSO-treated DENV, as detected by immunoblotting against the E protein, equilibrates near the bottom of a sucrose gradient, while exposure to pH 5.5 causes association with liposomes and equilibration near the top of the gradient. (*Second row*) DENV treated with 4G2, a mouse antibody targeted at the DENV fusion loop, does not associate with liposomes regardless of pH. Antibody against the capsid (C) protein was used to detect location of virus in fractions. (*Bottom two rows*) DENV pre-treated with 2-12-2 or 8-24-3 shows a pH-dependent sedimentation pattern similar to DMSO-treated virus.



**Figure 3-10. Compounds prevent completion of viral fusion with target membranes.**

Western blot of capsid protection assay. (A) At pH 8.0, capsid (C) protein can be detected via immunoblotting. Exposure to pH 5.5 causes loss of capsid signal in DMSO-treated virus, while signal is maintained when virus is pre-treated with 2-12-2 (concentrations: 5, 15, 25, 40  $\mu$ M). As a control for trypsin activity, virus and liposomes were treated with Triton X-100 (TX100) (B) DENV pre-treated with 8-24-3 (concentrations: 5, 15, 25  $\mu$ M). (C) DENV pre-treated with 111-2-B (concentrations: 10, 20, 40  $\mu$ M), a compound previously shown to be inactive in cellular assays. (D) Repeat of DENV with 2-12-2 (concentrations: 5, 15, and 25  $\mu$ M). DENV treated with 4G2, an antibody that recognizes the fusion loops, retains capsid signal, showing that capsid digestion is caused by fusion mediated by the E protein.

signal (Figure 3-10C). This strongly suggests that DENV2 does not complete fusion in the presence of 2-12-2 or 8-24-3. To confirm that degradation of the capsid protein is due to fusion mediated by the DENV E protein in this assay, we treated DENV with 4G2, an antibody that recognizes the viral fusion loops, and found retention of the capsid protein at pH 5.5 (Figure 3-10D).

These data suggest a model in which 2-12-2 and 8-24-3 prevent DENV entry by blocking a late step in the viral fusion cascade, thus preventing escape of the DENV capsid from the endosome and the delivery of the DENV genome to the cytoplasm for replication and productive infection of the host cell.

## **DISCUSSION**

In this chapter, we examine the mechanism by which two small molecules, 2-12-2 and 8-24-3, identified in an SAR study using GNF-2 as a scaffold, inhibit DENV entry. In addition to exploring the parameters of compound pre-incubation with virus inocula to learn more about how the compounds interact with the virus, we also investigated the biochemical mechanism of action of the compounds to determine what step of DENV entry they inhibit.

Pre-incubation of virus inocula with both 2-12-2 and 8-24-3 had to be carried out at 37°C in order to observe DENV inhibition (Figure 3-5), suggesting that the compounds' anti-DENV activity may be dependent on dynamic properties of the DENV envelope for transient exposure to binding site(s), either on the E protein itself or the virion lipid membrane. Significant inhibition of DENV infectivity by 2-12-2 and 8-24-3 required a short pre-incubation time with virus inocula (Figure 3-6), as no significant



reduction was observed when virus and compounds were added to cells simultaneously (Figure 3-2).

We hypothesize that 2-12-2 and 8-24-3 prevent a late step in the viral fusion cascade, since compound-treated DENV formed stable associations with liposomes (Figure 3-9) but could not complete fusion as measured by formation of the final fusion pore (Figure 3-10). The E protein is hypothesized to go through several intermediate, transient conformational forms between the pre-fusion dimer and the post-fusion trimer that involve movement or rotation of separate domains (Figure 1-3). Our results with 2-12-2 and 8-24-3 suggest that they block a step after insertion of the fusion loops into the target membrane, possibly the fold-back of domain III or association of the stem region with the trimer. One way to differentiate between these possibilities would be to examine whether hemifusion occurs when DENV is treated with the compounds. An *in vitro* assay measuring hemifusion of pyrene-labeled DENV with liposomes has previously been performed to test the activity of inhibitory peptides derived from the stem region of E (Schmidt et al., 2010). However, our attempts with this assay did not yield sufficient dynamic range to confidently detect hemifusion of DENV. Further optimization of this assay may be possible and would allow us to more exactly pinpoint the fusion step blocked by 2-12-2 and 8-24-3. Another possible approach to explore DENV fusion more precisely would be to test whether recombinant soluble E (sE) protein forms stable post-fusion trimers after incubation with compounds.

Further exploration of the mechanism of inhibition by 2-12-2 and 8-24-3 may reveal valuable experimental support for the hypothesized structural fusion intermediates, which currently is scarce for flaviviruses. Studies in alphaviruses, which also encode a

class II fusion protein, have confirmed an extended intermediate structure, in which the fusion loops are inserted into target membranes, and also revealed that this conformation is reversible (Roman-Sosa and Kielian, 2011). However, it is unclear if this is the case in DENV, since other studies have shown that domain I/II constructs of the glycoprotein in alphaviruses form stable trimers independent of domain III (Sánchez-San Martín et al., 2008), while DENV domain I/II trimers are not stable until exogenous domain III is added (Liao et al., 2010).

For this study, we chose two of the most potent compounds from the twenty-three compounds identified from a SAR study using small molecule GNF-2 as a scaffold. Our previous work showed that GNF-2 interacts directly with dengue virions and the DENV recombinant sE pre-fusion dimer (Chapter 2). We hypothesize that 2-12-2 and 8-24-3 also target the E protein but do not have direct experimental evidence to support this. A biotinylated derivative of 2-12-2 synthesized by the Gray lab exhibited a significant loss of activity when tested in cellular assays. The order of addition, MOI titration experiments, and the fact that the compounds inhibit fusion, a process mediated by E, strongly suggest that 2-12-2 and 8-24-3 target the virion or dengue E protein, although it is possible, though unlikely, that the compounds interact non-specifically with the lipid envelope of the virus. In addition, the location of binding on the E protein remains unknown for GNF-2, 2-12-2, and 8-24-3; even if the latter compounds interact with E, the binding sites may differ. Another open question is whether GNF-2, as well as other, untested compounds from the SAR study, block the same step of DENV entry as 2-12-2 and 8-24-3. Testing GNF-2 and the other analogs in the cellular and biochemical assays presented in this chapter would address this question.

One potential binding location on the E protein for GNF-2, 2-12-2, and 8-24-3 is the “hinge region” located between domains I and II. This site has previously been shown to be a ligand binding site, as crystallization of recombinant E revealed a single molecule of beta-octo-glucoside (BOG) in the pocket formed by domains I and II (Modis et al., 2003). Several other small molecule inhibitors of DENV entry have also been hypothesized to bind in this area (Poh et al., 2009; Schmidt et al., 2012; Wang et al., 2009). However, binding in this region was originally predicted to inhibit the extension of domain II of E away from the viral membrane and hence block the insertion of the fusion loops into a target membrane (Modis et al., 2003). Our experiments show that DENV treated with 2-12-2 or 8-24-3 makes stable associations with lipid bilayers, suggesting the block by these compounds occurs later in the fusion cascade. It is possible that binding in the domain I/II interface does not prevent fusion loop exposure, or the compounds may bind in a different location on the E protein. A previous study identified two other regions of the E protein that were predicted to be potential small molecule binding locations that prevent conformational changes of E that occur after insertion of the fusion (Yennamalli et al., 2009), so it is possible that our compounds could bind in either of these additional locations.

Overall, this chapter characterizes inhibition of DENV infectivity by two Abl-inactive small molecules that were identified in our SAR study. Using both cellular and biochemical assays, we have discovered that inhibition by 2-12-2 and 8-24-3 is temperature-dependent and lost when initial DENV MOI is increased. Neither compound blocked attachment of the virus to cells or prevented viral association with membranes, although they did prevent viral fusion pore formation with target membranes. Further

exploration of how these compounds inhibit DENV fusion may reveal valuable information about conformational intermediates of the E protein as it mediates viral fusion.

## **METHODS AND MATERIALS**

### **Order of addition experiments**

DENV2 (NGC strain) was used to infect BHK-21 cells in 24-well plates at an MOI of 1 for all experiments. Compounds were used at 5  $\mu$ M. Cells or virus were pre-incubated in 100  $\mu$ L medium containing compounds or DMSO at 37°C (cells: 1 hour; virus: 45 minutes) or 4°C (pre-incubation temperature; Figure 3-5). For co-infection, 5  $\mu$ M of either compound or DMSO was added to virus inoculum directly before addition to cells. After an initial one hour infection, cells were washed twice with 1X PBS to remove unbound compound and virus and 1 mL cell medium (MEM-alpha with 2% FBS) was added. For post-treatment of cells, compound was added to medium for 1 hour post-infection, then removed and cells were washed twice and medium was replaced. Twenty-four hours after initial infection, supernatant was collected and viral titer was determined by plaque-forming assay.

### **Removal of unbound compounds by gel filtration column**

DENV2 (NGC strain) inocula (MOI 2) was pre-incubated in 100  $\mu$ L with 10  $\mu$ M of compound or DMSO for 45 minutes at 37°C, then placed over a Sephadex G50 spin column and spun for 2 minutes at 2000 rpm at room temperature. Eluant was used to infect cells for 1 hour, after which cells were washed twice with 1X PBS to remove unbound compound and virus, and 1 mL cell medium (MEM-alpha with 2% FBS) was added. Twenty-four hours after initial infection, supernatant was collected, and viral titer was determined by plaque-forming assay.

### **Multiplicity of infection (MOI) titration**

DENV2 (NGC strain) inocula (various MOI) were pre-incubated with 5  $\mu$ M of compound or DMSO for 45 minutes at 37°C. After an initial one hour infection, cells were washed twice with 1X PBS to remove unbound compound and virus, and 1 mL cell medium (MEM-alpha with 2% FBS) was added. Twenty-four hours after initial infection, supernatant was collected, and viral titer was determined by plaque-forming assay.

### **Pre-incubation timecourse**

DENV2 (NGC strain) inocula (MOI 1) was pre-incubated with 5  $\mu$ M of either compound or DMSO for noted time at 37°C. Mixture was added to cells and left at 37°C for exactly one hour with gentle rocking. After the one hour infection, cells were washed twice with 1X PBS to remove unbound compound and virus, and 1 mL cell medium (MEM-alpha with 2% FBS) was added. Twenty-four hours after initial infection, supernatant was collected, and viral titer was determined by plaque-forming assay.

### **Reversibility experiments**

Due to the initial small volume of the virus, experiments were performed to determine the correct concentration of compounds to use in order to observe a consistent one-log drop in DENV titer. Five DENV  $\mu$ L ( $10^7$  pfu/ml) was incubated with 20  $\mu$ M of 2-12-2 or 8-24-3 or DMSO for 40 minutes at 37°C. Virus was diluted 100X and 200  $\mu$ L was used to infect BHK cells (effective MOI: 0.4) immediately or after an additional 40 minute incubation at 37°C. One hour after virus mixture was added to cells, cells were washed twice with 1X PBS to remove unbound compound and virus, and 1 mL cell medium (MEM-alpha with 2% FBS) was added. Twenty-four hours after initial infection,

supernatant was collected, and viral titer was determined by plaque-forming assay.

### **Plaque-forming assays**

To determine viral titer,  $10^5$  BHK-21 cells/well were plated in a 24-well plate to form a confluent monolayer. Ten-fold serial dilutions of culture supernatant were made in Earle's balanced salt solution (EBSS), and 100  $\mu$ L of the dilutions were used to infect cells for one hour at 37°C with gentle rocking, after which cells were washed once with 1X PBS. Overlay medium was added (1.05% CMC with MEM $\alpha$ , 2% FBS, Pen/Strep) and plates were incubated for 5 days (BHK-21). Assays were washed twice with 1X PBS to remove overlay medium, and cells were fixed with a mixture of 0.5% crystal violet and 3.7% formaldehyde. After a minimum of four hours, plates were washed, and plaques were counted.

### **DENV binding to cells**

DENV2 (NGC strain) was incubated with either 5  $\mu$ M of 2-12-2 or 8-24-3, 20  $\mu$ g/ml of heparin, or DMSO for 45 minutes at 37°C, then added to BHK cells (MOI 1) at 4°C for one hour with constant rocking. Cells were washed three times with cold 1X PBS to remove unbound virus or compound. For quantitation of viral genomes bound, total RNA was extracted using Trizol according to manufacturer's instructions. For viral yield determination, one mL of medium was added (MEM-alpha with 2% FBS), and culture supernatant was harvested 24 hours later. RT-qPCR was performed to determine number of DENV genomes present using primers against the C protein region of the genome. To determine viral titer in supernatant, plaque-forming assays were performed.

### **Quantification of DENV genomes**

Quantification of DENV genomes was done on a MyIQ Cyclor (Biorad). After RNA extraction and quantification, 5 µL of RNA was used to make cDNA with the iScript kit (Biorad) according to manufacturer's instructions. The resulting cDNA was digested by RNaseH for 1 hour at 37°C. Quantification by rt-PCR was done on each sample in triplicate (25 µL each) using SybrGreen Supermix (Biorad) according to manufacturer's instructions. Primers used targeted the DENV C gene.

DV2Cfor 5'-AATATGCTGAAACGCGAGAGA-3'

DV2Crev 5'-GGGATTGTTAGGAAACGAAGG-3'

### **Liposomes**

Liposomes were made with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) (Avanti Polar Lipids cat # 850457 and 850757, respectively), and cholesterol (Sigma-Aldrich cat#C3045) at 1:1:2 molar ratio in TAN buffer (20mM triethanolamine, 100mM NaCl, pH 8.0). Lipids were dried down and resuspended thoroughly by vortexing and sonication. Liposomes were prepared by extrusion through a 0.2 micron filter after five freeze/thaw cycles. For trypsin-containing liposomes, 10 mg of trypsin was added to 1 mL of lipids (3 mg each) after the third freeze/thaw cycle, prior to extrusion. Liposomes were separated from unincorporated trypsin by size-exclusion chromatography using a Superdex 200 column on an Akta fast performance liquid chromatography (FPLC) system. Trypsin-containing liposomes were used within 24 hours; liposomes for coflotation were used within three days.



### **Coflotation assay**

Purified dengue 2 virus (600 ng as quantified by Bradford assay) was incubated with 40  $\mu$ M of compounds or DMSO for 45 minutes at 37°C in 200  $\mu$ L TAN buffer (pH 8.0). Liposomes (20  $\mu$ L) were added for ten minutes, followed by lowering of the pH by addition of 15  $\mu$ L 1M sodium acetate (pH 5.0). Following a ten minute incubation, samples were back-neutralized with 30  $\mu$ L 1M TEA (unbuffered). Samples were then mixed with 25% sucrose in TAN buffer (pH 8.0) and placed into a four step gradient (60%, 25%, 15%, 5% sucrose in TAN buffer) and spun for 3 hours at 44,000 rpm. Gradients were fractionated and 20% TCA (total volume) was added to precipitate proteins. Samples were washed twice with 1 mL acetone, and sample buffer was added. Fractions were boiled for 20 minutes and run on a 10% SDS-PAGE gel followed by semi-dry transfer to a nitrocellulose membrane. 4G2 antibody, grown in house from ATCC cell line HB-112, was used for detection of DENV envelope (dilution varies by lot), and the neat supernatant of hybridoma cell line 6F3.1, kindly provided by Dr. John Aaskov, was used to detect capsid protein.

### **Capsid protection assay**

Purified dengue 2 virus (300 ng) was incubated with various concentrations of compounds (5-40  $\mu$ M) for 45 minutes at 37°C in TAN buffer (pH 8.0) prior to addition of liposomes (7.5  $\mu$ L of peak fraction from column) for ten minutes. After incubation with liposomes, 7.5  $\mu$ L 1M sodium acetate (pH 5.0) was added to drop pH to 5.5 for ten minutes. Samples were back-neutralized with 15  $\mu$ L 1M TEA (unbuffered) and incubated for forty-five minutes at 37°C to allow trypsin digestion (total volume: 100  $\mu$ L). SDS sample buffer (20  $\mu$ L) was added, and samples were boiled for 20 minutes before being

run on a 12% SDS-PAGE gel to separate proteins. Proteins were transferred to a nitrocellulose membrane using a semi-dry transfer. 4G2 antibody, grown in house from ATCC cell line HB-112, was used for detection of DENV envelope (dilution varies by lot), and the neat supernatant of hybridoma cell line 6F3.1, kindly provided by Dr. John Aaskov, was used to detect capsid protein.

## REFERENCES:

- Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., and Marks, R.M. (1997). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* 3, 866-871.
- Harrison, S.C. (2005). Mechanism of Membrane Fusion by Viral Envelope Proteins. In *Advances in Virus Research*, R. Polly, ed. (Academic Press), pp. 231-261.
- Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., *et al.* (2002). Structure of Dengue Virus: Implications for Flavivirus Organization, Maturation, and Fusion. *Cell* 108, 717-725.
- Liao, M., Martín, C.S.-S., Zheng, A., and Kielian, M. (2010). In Vitro Reconstitution Reveals Key Intermediate States of Trimer Formation by the Dengue Virus Membrane Fusion Protein. *Journal of Virology* 84, 5730-5740.
- Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America* 100, 6986-6991.
- Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature* 427, 313-319.
- Mukhopadhyay, S., Kuhn, R.J., and Rossmann, M.G. (2005). A structural perspective of the flavivirus life cycle. *Nat Rev Micro* 3, 13-22.
- Poh, M.K., Yip, A., Zhang, S., Priestle, J.P., Ma, N.L., Smit, J.M., Wilschut, J., Shi, P.-Y., Wenk, M.R., and Schul, W. (2009). A small molecule fusion inhibitor of dengue virus. *Antiviral Research* 84, 260-266.
- Roman-Sosa, G., and Kielian, M. (2011). The Interaction of Alphavirus E1 Protein with Exogenous Domain III Defines Stages in Virus-Membrane Fusion. *Journal of Virology* 85, 12271-12279.
- Sánchez-San Martín, C., Sosa, H., and Kielian, M. (2008). A Stable Prefusion Intermediate of the Alphavirus Fusion Protein Reveals Critical Features of Class II Membrane Fusion. *Cell Host & Microbe* 4, 600-608.
- Schmidt, A.G., Lee, K., Yang, P.L., and Harrison, S.C. (2012). Small-Molecule Inhibitors of Dengue-Virus Entry. *PLoS Pathog* 8, e1002627.
- Schmidt, A.G., Yang, P.L., and Harrison, S.C. (2010). Peptide Inhibitors of Dengue-Virus Entry Target a Late-Stage Fusion Intermediate. *PLoS Pathog* 6, e1000851.

van der Schaar, H.M., Rust, M.J., Waarts, B.-L., van der Ende-Metselaar, H., Kuhn, R.J., Wilschut, J., Zhuang, X., and Smit, J.M. (2007). Characterization of the Early Events in Dengue Virus Cell Entry by Biochemical Assays and Single-Virus Tracking. *Journal of Virology* 81, 12019-12028.

Wang, Q.-Y., Patel, S.J., Vangrevelinghe, E., Xu, H.Y., Rao, R., Jaber, D., Schul, W., Gu, F., Heudi, O., Ma, N.L., *et al.* (2009). A Small Molecule Dengue Virus Entry Inhibitor. *Antimicrob Agents Chemother*, AAC.01148-01108.

WHO (2012). Dengue and severe dengue.

Yennamalli, R., Subbarao, N., Kampmann, T., McGeary, R., Young, P., and Kobe, B. (2009). Identification of novel target sites and an inhibitor of the dengue virus E protein. *Journal of Computer-Aided Molecular Design*.

**Chapter 4:** Exploring potential resistance mutations in DENV structural proteins against small molecule entry inhibitors

**Acknowledgments:** I would like to thank Dr. Ted C. Pierson of the NIH for generously supplying reagents and protocols for the recombinant viral particle system. I would also like to thank members of the Gray lab for synthesizing the small molecule entry inhibitors, and members of the Yang lab for discussion and advice.

## SUMMARY

In this chapter, we examined the effect(s) of amino acid changes in the DENV structural proteins on susceptibility of the virus to small molecule entry inhibitors. We serially passaged DENV2 in the presence of select compounds and tracked viral titer to monitor the emergence of inhibitor-resistant DENV. This approach yielded a rise in titer against one out of ten tested compounds, 7-148-6. Sequencing of the structural proteins from this virus revealed two changes: one each in the E protein, E(M196V) and M protein, M(H28Y). Testing of these individual residues in a single-cycle infection assay using recombinant viral particles (RVPs) (Wang et al., 2009a) revealed that E(M196V) enhanced entry of virus particles, while M(H28Y) decreased the efficiency of entry. RVPs containing either change showed no significant decrease in reporter signal when pretreated with 7-148-6. E(M196V) is located in a “hinge” region between domains I and II of the E protein that has been hypothesized to be critical for conformational rearrangements necessary to mediate membrane fusion (Modis et al., 2003). Using the RVP system, we performed site-directed mutagenesis of eleven selected residues in this region and found that eight mutations decreased production or impaired entry of RVPs. We focused on two mutations, E(Q52A) and E(Q131A), and found that they conferred resistance against three small molecule entry inhibitors. Overall, our data suggest changes in the E protein domain I/II interface may confer resistance to small molecule entry inhibitors and affect production or release of viral particles as well as their entry into target cells.

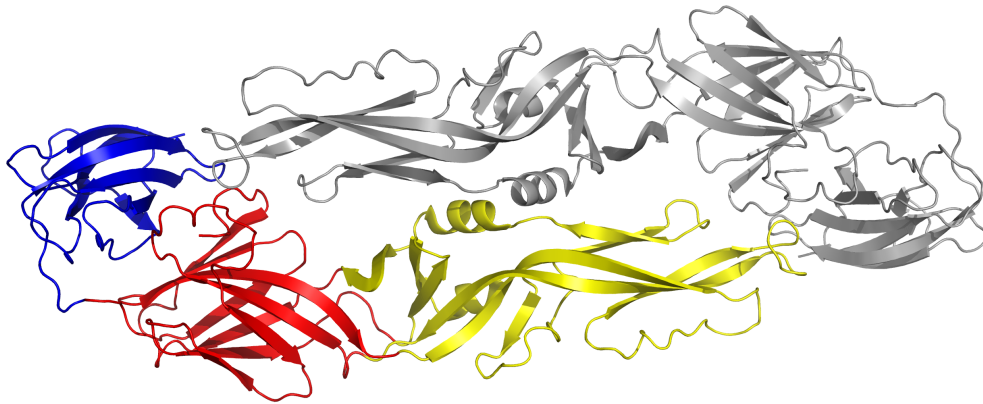
## INTRODUCTION

Dengue virus (DENV) is one of the most common vector-borne viruses in the world, causing an estimated 50 to 100 million illnesses per year (WHO, 2012). There are no specific therapeutics or vaccines available against DENV, making antivirals to treat the disease highly desirable. Virus entry is considered an attractive antiviral target in part because blocking entry can prevent primary infection or viral spread. For DENV, virus entry is mediated by the envelope (E) protein, the major glycoprotein on the virion surface.

DENV E consists of three domains (Figure 4-1) and mediates binding to cells as well as fusion with the cellular membrane, which is triggered by low pH inside endosomes after DENV is endocytosed (Gollins and Porterfield, 1986). Domain I is centrally located, while finger-like domain III extends along the virion surface and contains the fusion loop at its distal end, and domain II has an immunoglobulin-like fold that contains the purported receptor-binding region (Mukhopadhyay et al., 2005). When the E protein is first synthesized into the ER lumen, it associates with prM, a protein that acts as a chaperone for E, and forms a heterodimer. This E/prM heterodimer is found on the surface of newly-assembled virions in the endoplasmic reticulum as trimeric spikes (Zhang et al., 2003b). As virions pass through the trans-Golgi network, E reassociates into homodimers, and the pr peptide is cleaved by furin, leaving the small M protein resting underneath the E dimers on the now-mature virion surface (Zhang et al., 2003a).

It is hypothesized that the domain I/II interface of E acts as a hinge region during viral fusion, allowing extension of the fusion loop towards the cellular lipid membrane (Modis et al., 2004). The region between domains I and II of the E protein is often





**Figure 4-1. Crystal structure of the DENV2 envelope protein.** DENV2 envelope (E) protein as a homodimer, the pre-fusion form found on the surface of mature virions. Domains I, II, and III of one E monomer are shown in red, yellow and blue, respectively. PDB ID: 1OAN (Modis et al., 2003)

referred to as the “BOG” pocket, as it was discovered to contain a single molecule of beta-octo-glucoside (BOG) under certain crystallization conditions (Modis et al., 2003). In high-resolution crystal structures, the pocket adopts a closed conformation with the *k-l* loop covering an opening between the domains when BOG is present but exhibits an open pocket due to shifting of the *k-l* loop when BOG is absent. Based on this observation and previous studies indicating that residues lining this region may alter the pH threshold of fusion (Beasley and Aaskov, 2001; Lee et al., 1997), it was proposed that the presence of a small molecule ligand in the BOG pocket may interfere with DENV entry by preventing the structural rearrangements involved in DENV fusion. An attractive feature of this proposal is that any mutations in this region that could confer resistance would be expected to decrease the fitness of the virus due to their potential effect(s) on fusion.

Since this potential strategy for inhibiting DENV was proposed, several inhibitors of DENV entry hypothesized to bind in the BOG pocket have been reported (Kampmann et al., 2009; Poh et al., 2009; Schmidt et al., 2012; Wang et al., 2009b); however, structural data directly demonstrating this has yet to be published. Likewise, genetic data mapping resistance mutations to this or any other region of the E protein have not been reported, although whether this is due to the effect of such mutations on E’s function and the fitness of the corresponding virus remains unclear.

Previously, we identified several small molecules that inhibited DENV when pre-incubated with virus inocula prior to initial infection of cells. We hypothesized that these compounds act on a viral target, specifically the envelope protein on the surface of virions, and showed that select compounds inhibited DENV fusion *in vitro*. In this study,

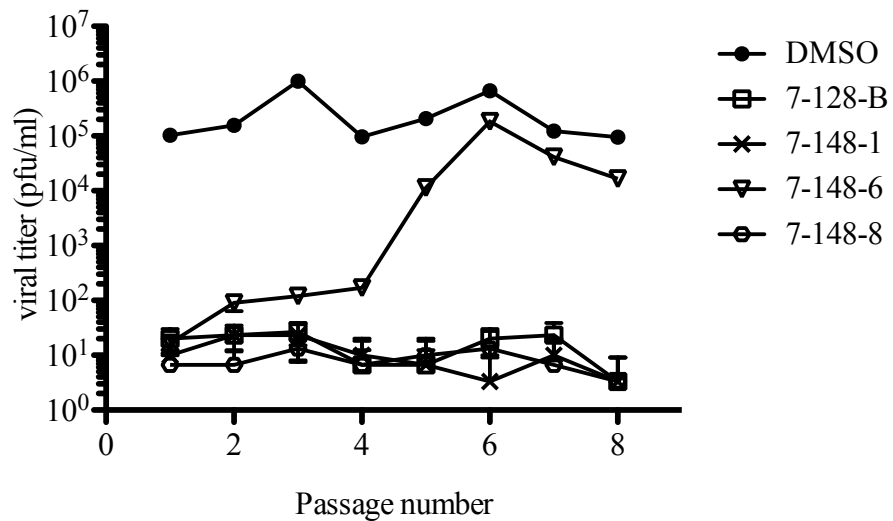
we report efforts to learn more about the molecular interactions of these inhibitors with the dengue virion by using mutational analysis to explore the molecular target of the inhibitors and potential mechanisms of resistance to these inhibitors. We serially passaged DENV2 in the presence of ten select compounds and monitored for outgrowth of DENV that would suggest viral resistance. We observed outgrowth of DENV in only one out of ten compound treatments, with small molecule 7-148-6. Consensus sequencing of the structural proteins of this resistant quasispecies led us to the identification of two point mutations, one in the domain I/II of the E protein, E(M196V), and one in the ectodomain of M, M(H28Y). We found that in a single-cycle DENV reporter assay, the presence of either mutation was sufficient to confer insensitivity to treatment of virus particles with 7-148-6. In parallel, we performed a rational, site-directed mutagenesis study of eleven residues around the domain I/II interface of E. We found that eight of these changes drastically reduced virus particle production or entry. We tested two amino acid mutations that were still permissive for efficient RVP entry, Q52A and Q131A, against select small molecule entry inhibitors GNF-2, 2-12-2, and 7-148-6, and found both the presence of either mutation reduced sensitivity to treatment relative to wild-type RVPs. Taken together, our data suggest that the DI/DII interface is sensitive to many amino acid changes; however, changes that are tolerated may decrease sensitivity to the small molecule entry inhibitors examined in these experiments. The results presented here aid in understanding the interaction of our small molecule inhibitors with the DENV E protein and also suggest that point mutations at the interface of domains I and II of E affect viral particle production and entry.

## RESULTS

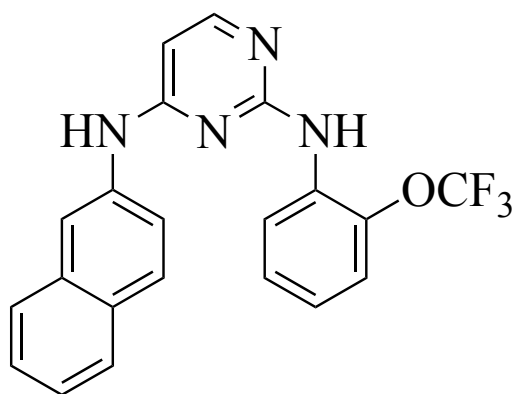
### Potential resistance of DENV2 to presence of small molecule 7-148-6

To examine the potential development of resistance to a subset of our small molecule entry inhibitors, we serially passaged virus in the presence of compounds using two approaches. We first tried passaging DENV2 in the presence of 20  $\mu$ M of four small molecules (7-128-B, 7-148-1, 7-148-6, and 7-148-8) previously shown to inhibit DENV infection yet inactive against intracellular Abl kinases (Appendix A). Over the course of eight passages, we saw a rise in viral titer suggestive of a resistant virus strain against only one of the compounds, 7-148-6 (Figure 4-2). Concerned that the high initial concentration of compounds had prevented outgrowth of resistant DENV, we selected another six compounds, 1-100-1, 2-12-2, 2-21-2, 8-24-3, 8-24-5, and 2-119-2-B, for a second serial passage. We gradually increased compound concentrations from 2.5 to 20  $\mu$ M over the course of nine passages, but found that viral titers crashed as the EC<sub>90</sub> values of compounds were reached and did not recover (data not shown). Thus, out of ten compounds tested, we identified viral outgrowth against only one, suggesting that DENV may not easily develop resistance to the small molecule entry inhibitors.

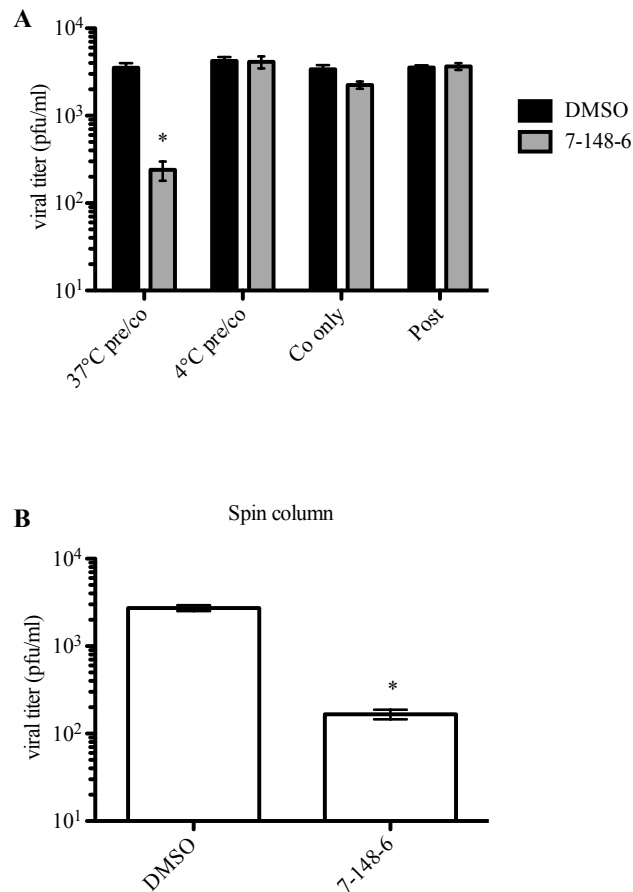
We turned our attention to compound 7-148-6 (Figure 4-3) and confirmed that it mirrored the activity of our previously tested compounds, 2-12-2 and 8-24-3, in the order of addition experiments and that it exhibited anti-DENV activity even after size-exclusion chromatography was used to remove unbound compound from the inoculum prior to infection of cells (Figure 4-4). We tested 7-148-6 in biochemical mechanism assays and found that it does not inhibit attachment of DENV to cells but does block completion of DENV fusion *in vitro* (Figure 4-5). We hypothesize that 7-148-6 inhibits



**Figure 4-2. Passaging of DENV2 in the presence of selected compounds.** DENV2 was passaged every four days in Vero cells in the presence of DMSO or 20  $\mu$ M of each compound. DENV titer was determined by plaque-forming assay. Each point represents the mean of three replicates with error bars showing standard deviation.

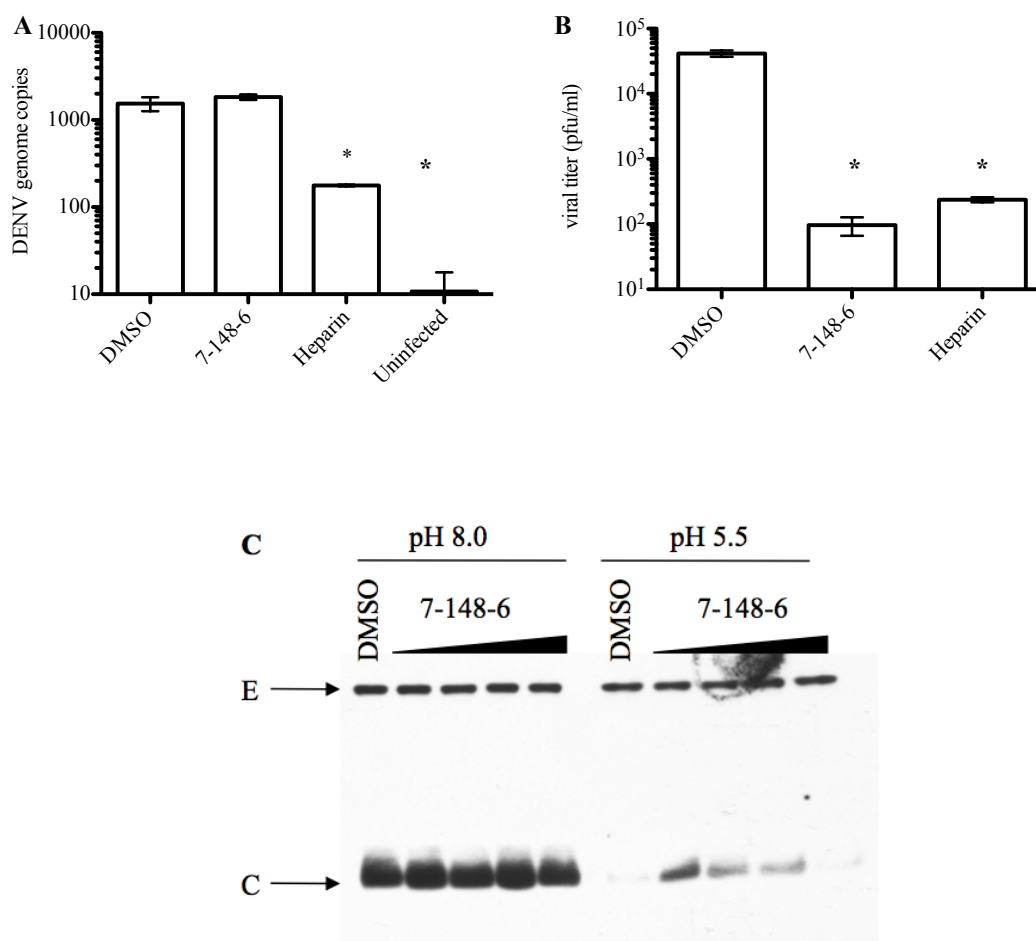


**Figure 4-3. Structure of compound 7-148-6.** EC<sub>90</sub> values against DENV serotypes in infectivity assay: DENV1 – 8  $\mu$ M; DENV2 – 10  $\mu$ M; DENV3 >40  $\mu$ M; DENV4 >40  $\mu$ M.



**Figure 4-4. Compound 7-148-6 inhibits DENV titer when pre-incubated with virus inoculum.** (A) Order of addition experiments show a significant reduction in viral yield only when compound 7-148-6 (10  $\mu$ M) is pre-incubated with DENV2 inoculum at 37°C. (B) Significant inhibition of DENV infectivity by 7-148-6 is observed when free compound was removed via a gel filtration spin column prior to cellular infection. Each bar represents the mean of three replicates with error bars showing standard deviation.

\* indicates p value < 0.01



**Figure 4-5. Compound 7-148-6 does not affect virus attachment to cells and prevents completion of DENV fusion.** DENV was incubated with 7-148-6 (10  $\mu$ M), DMSO, or heparin sulfate (20  $\mu$ g/ml) for 45 minutes at 37°C, after which mixture was added to chilled cells for 1 hour at 4°C. After washing, (A) total RNA was extracted for qRT-PCR or (B) infection was allowed to proceed for 24 hours and viral yield was measured by PFA. Each bar represents the mean of three replicates with error bars showing standard deviation. \* indicates  $p$  value < 0.01 (C) Western blot against the DENV E and C proteins shows retention of the C signal when purified DENV is treated with 7-148-6 (10, 20, 30, 40  $\mu$ M) prior to exposure to pH 5.5 in the presence of trypsin-containing liposomes.

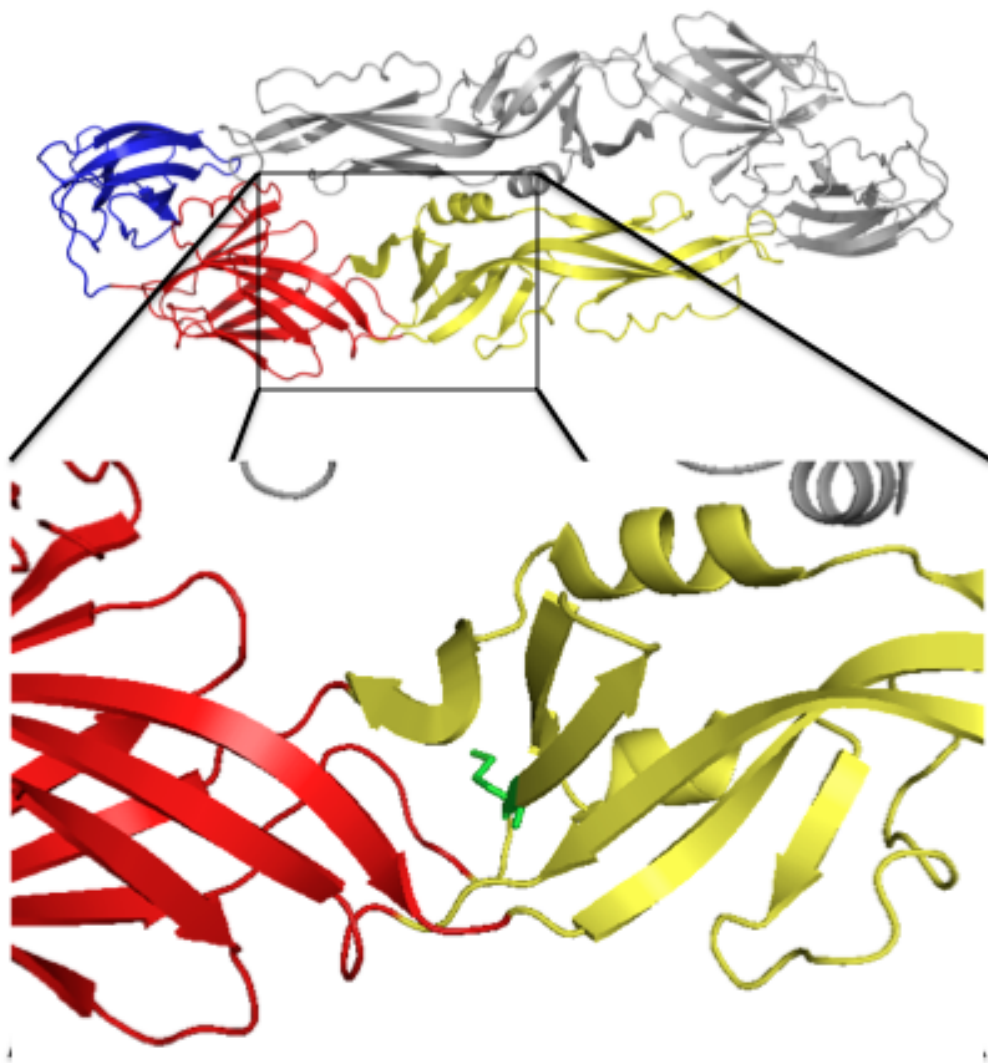


DENV entry by blocking a late step in DENV fusion via interactions with the E protein on the virion surface. These results confirm that 7-148-6 behaves similarly to our other tested compounds, 2-12-2 and 8-24-3 (Chapter 3), and can be used as a tool to explore DENV resistance against the small molecule entry inhibitors.

### **Sequencing of resistant virus reveals two changes in the DENV structural proteins**

In order to identify what sequence change(s) caused the observed rise in DENV2 titer, we extracted viral RNA from passage 6 of the 7-148-6 passaged virus (Figure 4-2). Consensus sequencing of the viral structural proteins C, prM, and E revealed a single amino acid change in the DENV2 envelope protein at position 196, from methionine to valine (Figure 4-6). This E(M196V) change was not observed in DENV2 that had been passaged in the presence of DMSO. Residue 196 is located at the base of the BOG pocket located between domains I and II, a region of the protein that has been hypothesized to act as a hinge during the conformational changes that occur during viral fusion (Modis et al., 2003), consistent with our hypothesis that the anti-DENV activity of 7-148-6 is due to inhibition of DENV fusion. The methionine at this position is conserved across all four DENV serotypes although it is not present in more distantly related flaviviruses such as West Nile or Japanese encephalitis virus.

Our consensus sequencing, while identifying E(M196V), also revealed that this change was incomplete. This raised the possibility that consensus sequencing may have obscured other changes in the structural genes, so we performed a single round of virus plaque isolation, expansion, and sequencing. This viral sequencing led to the identification of an additional amino acid change, present in 6/7 plaques of the 7-148-6



**Figure 4-6. Location of residue 196 on DENV2 E protein.** Pre-fusion envelope dimer crystal structure (Modis et al., 2003). Magnification is of the domain I (red) – domain II (yellow) interface. (*Inset*) The methionine at position 196 is highlighted in green. In DENV2 serially passaged in the presence of 20  $\mu$ M of 7-148-6, consensus sequencing revealed a valine at this position.

passaged virus and 0/3 of virus plaques passaged in the presence of DMSO. This change was a histidine to tyrosine mutation located at residue 28 in the ectodomain of the M protein. The M protein is the post-cleavage remnant of the chaperone protein prM, and no functional role has yet been described for M in the viral entry process. A study that used electron density to map the location of M on the surface of the mature dengue virion suggests that the ectodomain takes the form of two alpha helices, one of which is amphipathic and makes contact with the lipid bilayer of the virion and possibly the underside of the E protein as well (Kuhn et al., 2002); M(H28Y) is hypothesized to be located in this helix.

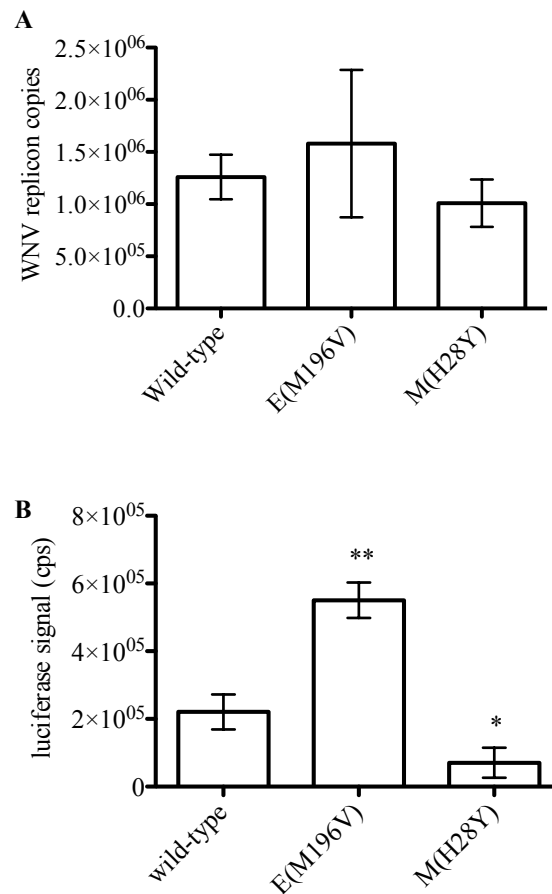
### **Effect of structural protein mutations on production and entry of single-cycle reporter virus**

To investigate the potential of each identified amino acid change to confer resistance, we used a reporter virus particle (RVP) system (Ansarah-Sobrinho et al., 2008). The RVP system uses two plasmids to produce single-cycle infectious viral particles, with luciferase signal as the readout of a successful infection. One plasmid encodes for DENV structural proteins C, prM, and E, while the second encodes a West Nile virus (WNV) genome in which structural proteins have been replaced with the *Renilla* luciferase gene. Transfection of cells with both plasmids allows for the production of recombinant virus particles (RVPs) comprised of the DENV structural proteins and containing a WNV RNA genome. Entry of RVPs is mediated by the DENV E protein on the particle surface and is thought to occur via the same mechanisms as authentic DENV. Following entry, the WNV RNA is translated and replicated, and

quantification of the resulting luciferase activity provides a measure of the steady-state abundance of WNV RNA. Accordingly, to examine the effects of the E(M196V) and M(H28Y) mutations on RVP production and entry, we engineered each mutation into the plasmid encoding the DENV2 CprME genes and used these plasmids to produce RVPs.

We initially determined the effect of E(M196V) or M(H28Y) on RVP production by quantifying the number of WNV replicons present in producer cell supernatant by qRT-PCR (Figure 4-7A). No significant difference in genome copy number was detected for either mutation when compared to wild-type RVP production. It is important to note that quantification of WNV genomes measures the release of recombinant viral particles that encapsidate RNA, but does not measure any potential differences in empty particle release. Unfortunately, due to the low number of RVPs in the supernatant, our attempts to detect any changes in the release of E protein or empty particles by transfected cells were unsuccessful.

We next asked if E(M196V) or M(H28Y) affected entry of RVPs into target cells. Wild-type, E(M196V), or M(H28Y) RVPs were diluted to normalize for WNV genome copy number and added to target cells for a one hour initial infection. Cells were washed to remove unbound RVPs and fresh medium was added. Sixteen hours after infection, cells were lysed, and luciferase activity was determined. We observed that E(M196V) RVPs had a significant increase in luciferase signal compared to wild-type RVP signal, while M(H28Y) RVPs had a significant decrease (Figure 4-7B). These data show that both mutations impact entry of viral particles, although in different directions.



**Figure 4-7. DENV structural protein mutations affect virus entry in a single-cycle infection assay.** Recombinant viral particles (RVPs) containing either E(M196V) or H(28Y) were produced by transfection of 293T cells with two plasmids: one encoding the WNV luciferase reporter replicon and one encoding the DENV structural cassette.

(A) RVPs in producer cell supernatant were quantified by performing qRT-PCR against the WNV replicon. Each bar represents the mean of three replicates with error bars showing standard deviation. (B) After quantification, RVPs were diluted to normalize for WNV copy number and BHK-21 cells were infected. Sixteen hours post-infection, cells were lysed and luciferase signal was measured. Each bar represents the mean of four replicates with error bars showing standard deviation. \*\*p value <0.001, \*p value <0.05

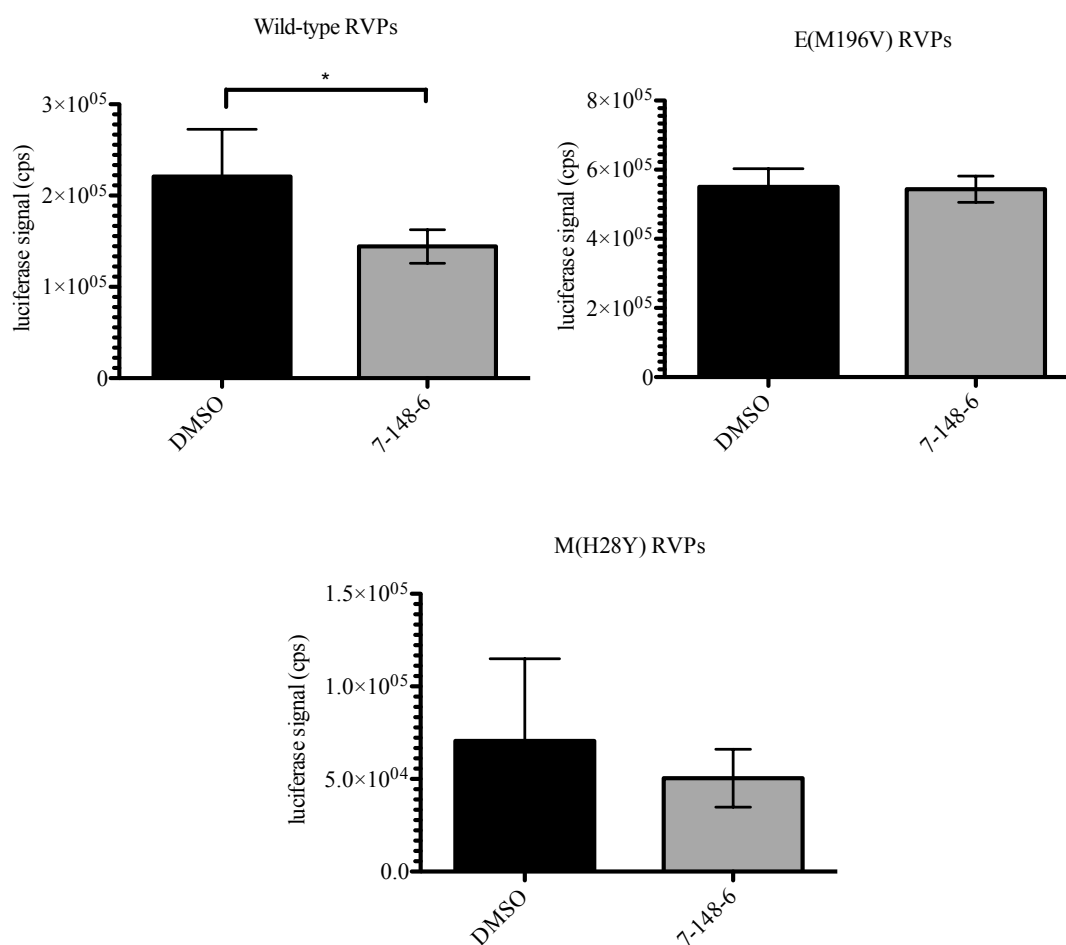
### **Resistance of DENV structural protein mutations to 7-148-6**

To examine the effect of E(M196V) and M(H28Y) mutations on sensitivity to compound 7-148-6, we normalized inocula by WNV genome copy number and treated RVPs with 7-148-6 prior to cell infection. Wild-type RVPs treated with 7-148-6 showed a significant decrease in luciferase signal when cells were lysed 16 hours post-infection and compared to DMSO-treated RVP signal (Figure 4-8). The luciferase signal produced by cells infected with E(M196V) RVPs did not decrease when RVPs were pre-treated with 7-148-6, indicating that E(M196V) confers resistance to 7-148-6. M(H28Y) RVPs did not have a significant decrease in luciferase signal after 7-148-6 treatment either, although the low overall luciferase activity of this mutant makes this result difficult to interpret.

These results suggest that the E(M196V) change may be responsible for the insensitivity of our passaged virus to the presence of 7-148-6. It is possible that M(H28Y) also contributes to resistance, and in the authentic virion, both mutations may contribute to resistance to 7-148-6. These data show that a single point mutation in the DENV E protein, even a conservative one from methionine to valine, enhances DENV entry and may alter sensitivity to a small molecule entry inhibitor.

### **Probing DENV envelope protein changes with single-cycle infection reporter virus**

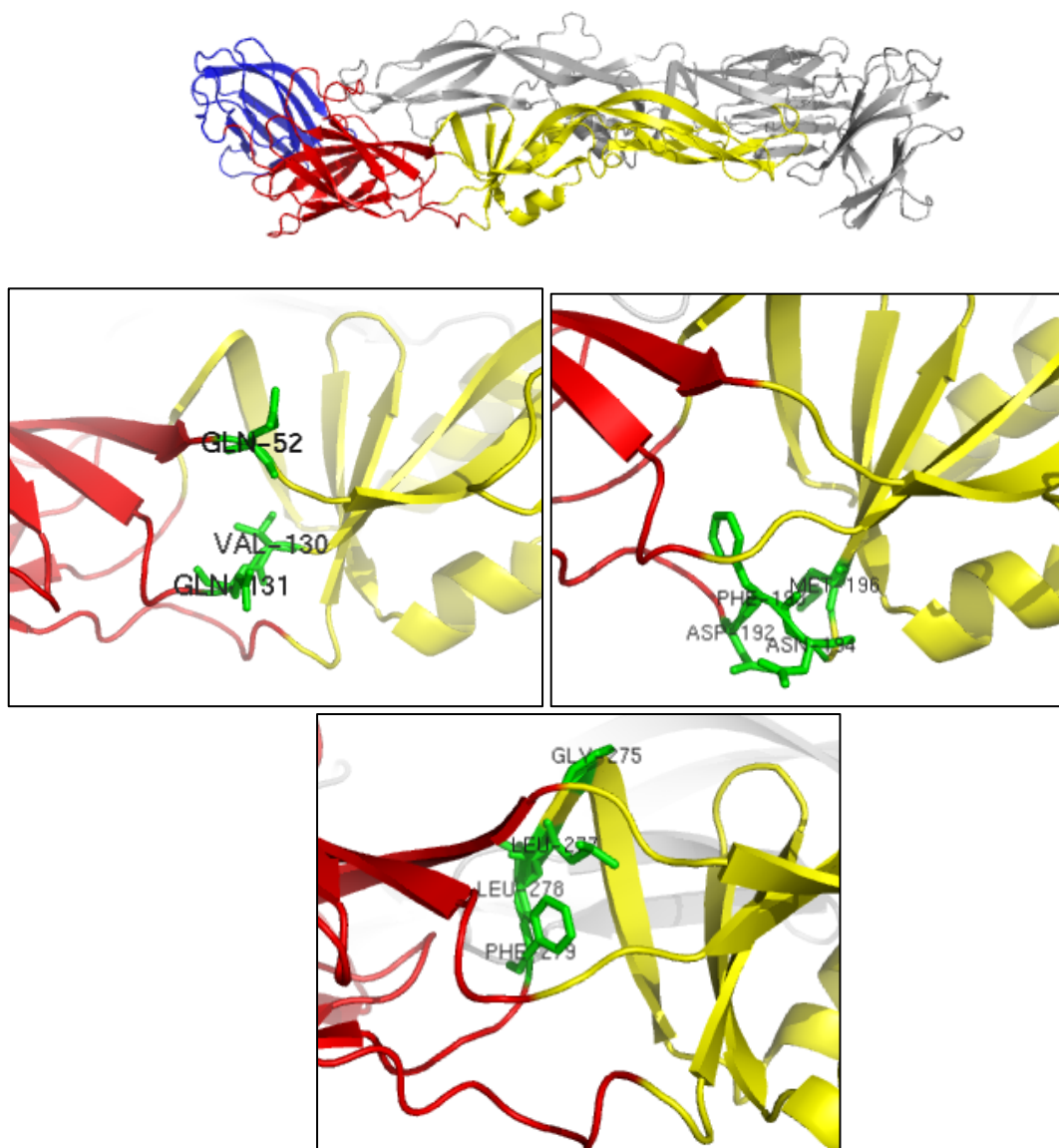
Serially passaging DENV in the presence of ten compounds revealed outgrowth of only one resistant virus. This suggests that it may be difficult for DENV to overcome the presence of small molecule entry inhibitors, possibly because any mutations that confer resistance may come at a high fitness cost for the virus. Since E(M196V) is



**Figure 4-8. RVPs with DENV structural protein changes show decreased sensitivity to compound 7-148-6.** Recombinant viral particles (RVPs) encoding wildtype structural proteins, E(M196V), or M(H28Y) mutant proteins were diluted to normalize for genome copy and subsequently used to infect BHK-21 cells after pre-treatment with 10  $\mu$ M compound 7-148-6. Cells were lysed 16 hours post-infection, and luciferase signal was measured. Each bar represents the mean of four replicates with error bars showing standard deviation. \*p value < 0.05

located at the base of the BOG pocket, an area of the protein thought to act as a “hinge region” for the large conformational change of E as it mediates membrane fusion (Modis et al., 2003), we wanted to probe the tolerance of substitutions in this region more directly. Using site-directed mutagenesis of the plasmid encoding the DENV structural genes (the “CprME plasmid”), we mutated each of eleven selected amino acids to alanine and tested production and entry in the RVP system (Figure 4-9). The chosen residues were selected due to either conservation among DENV serotypes or previous implication in altering the optimal pH of fusion, and mapped to three regions of the BOG pocket. Three residues, Q52, V130, and Q131 are clustered around the bottom outer edge of the pocket. V130 is conserved across DENV serotypes, while both Q52 and Q131 have been previously implicated to alter the optimal pH of fusion (Modis et al., 2003). Another four residues, D192, F193, N194, and M196 are found in a sequence, <sup>192</sup>DFNEM<sup>196</sup>, which is conserved across DENV serotypes; these residues form the bottom of the BOG pocket and sit close to the lipid membrane of the virion. This region forms a pseudo-alpha helix that may make inter-trimer contacts in the post-fusion trimer structure (Modis et al., 2004) and, outside of the fusion loop, is one of the largest stretches of conserved amino acids in the DENV envelope protein. M196 is also the residue altered to valine in the passaging of DENV in the presence of 7-148-6, and we were curious to determine the effect of an alanine at this position. The final set of amino acids chosen, G275, L277, L278, and F279 are not part of the pocket itself, but rather located on the *k-l* loop that covers the opening of the pocket. G275, L277, and F279 are conserved across DENV serotypes, while F279 has also been suggested to alter optimal pH of fusion (Lee et al., 1997).





**Figure 4-9. Location of selected residues for mutagenesis on the DENV2 envelope crystal structure.** Crystal structure of DENV envelope protein (Modis et al., 2003).

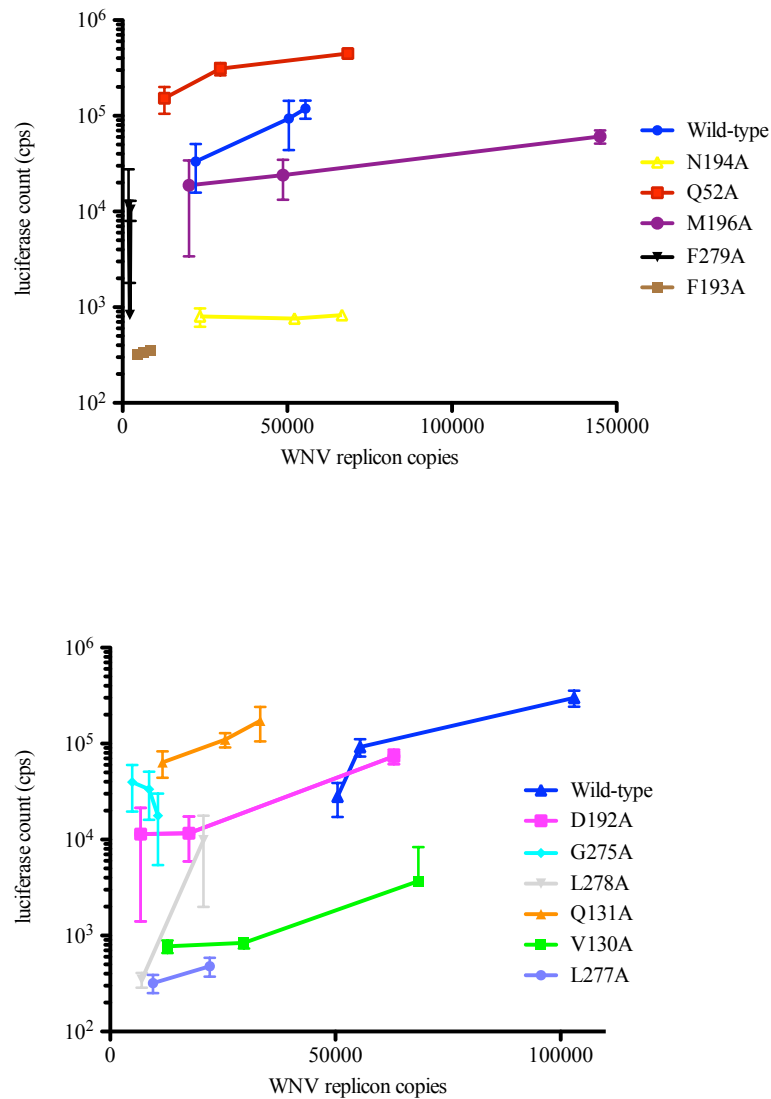
(*Top*) Pre-fusion DENV E dimer. Each inset focuses on a group of amino acids selected for mutagenesis. (*Middle left*) Q52, V130, and Q131, all located near the front and bottom of the BOG pocket. (*Middle right*) D192, F193, N194, and M196, part of the chain that forms the bottom of the BOG pocket (*Bottom*) G275, L277, L278, F279, all located on the *k-l* loop.

We mutated each residue to an alanine in the CprME plasmid and used these plasmids to transfect cells to produce RVPs. RVP preparations were titrated by quantifying the number of WNV genomes/mL present in serial dilutions of producer cell supernatant. We graphed this value against luciferase activity detected in target cells 16 hours post-infection, allowing us to draw a picture of the effect of mutations on both RVP production and entry (Figure 4-10). We found that three mutations, V130A, N194A, and L277A, had detectable levels of WNV genomes in producer cell supernatant but low luciferase activity in target cells. This suggests that these changes may have a detrimental effect on RVP entry. In contrast, mutation to an alanine at positions 193 or 279, originally phenylalanines, caused low luciferase activity in RVP target cells but also near undetectable levels of WNV genomes in producer cell supernatant, suggesting these change may impact production of RVPs.

The methionine to valine and methionine to alanine mutations at position 196 differed in their qualitative effects on RVP entry, which may suggest that loss of hydrophobic bulk at this site has a deleterious effect on this viral process. While more extensive mutagenesis of this region is necessary to validate this hypothesis, our current results demonstrate that mutations in the E protein “hinge region” can affect both production and entry of virus particles.

### **Changes in the DI/DII region may affect sensitivity to small molecule entry inhibitors**

We selected two mutations, Q52A and Q131A, from our RVP screen to test against small molecule entry inhibitors to determine if these changes conferred resistance.



**Figure 4-10. Changes in the DENV E protein DI/DII interface affect production and entry of RVPs.** RVPs with alanines introduced at select locations were produced and qRT-PCR was performed on serial dilutions to determine RVP production (x axis). Serially diluted supernatants were used to infect BHK-21 cells, and lysate was collected 16 hours post-infection to detect luciferase activity as a measure of RVP entry (y axis). Each point represents the mean of four replicates with error bars showing standard deviation.

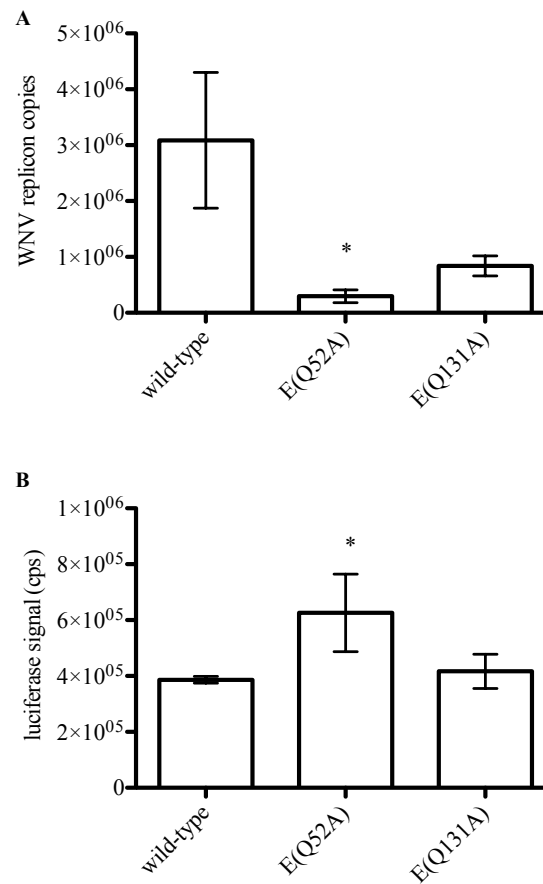
Examination of our RVP screen suggested that these two mutants may enter cells more efficiently than the wild-type, a phenotype similar to that of E(M196V). Thus, we hypothesized that the increased infectivity of Q52A and Q131A might also be associated with resistance to our compounds.

As we had done for E(M196V) and M(H28Y), we first measured E(Q52A) and E(Q131A) RVP production by quantifying WNV genome in producer cell supernatant (Figure 4-11A). We found that production of E(Q52A) RVPs was significantly reduced compared to wild-type RVPs. However, when we measured luciferase activity after normalizing to genome number, we found that E(Q52A) RVPs actually had a significant increase in luciferase signal (Figure 4-11B). These results suggest that the E(Q52A) mutation may have a negative impact on viral particle production but enhance viral entry.

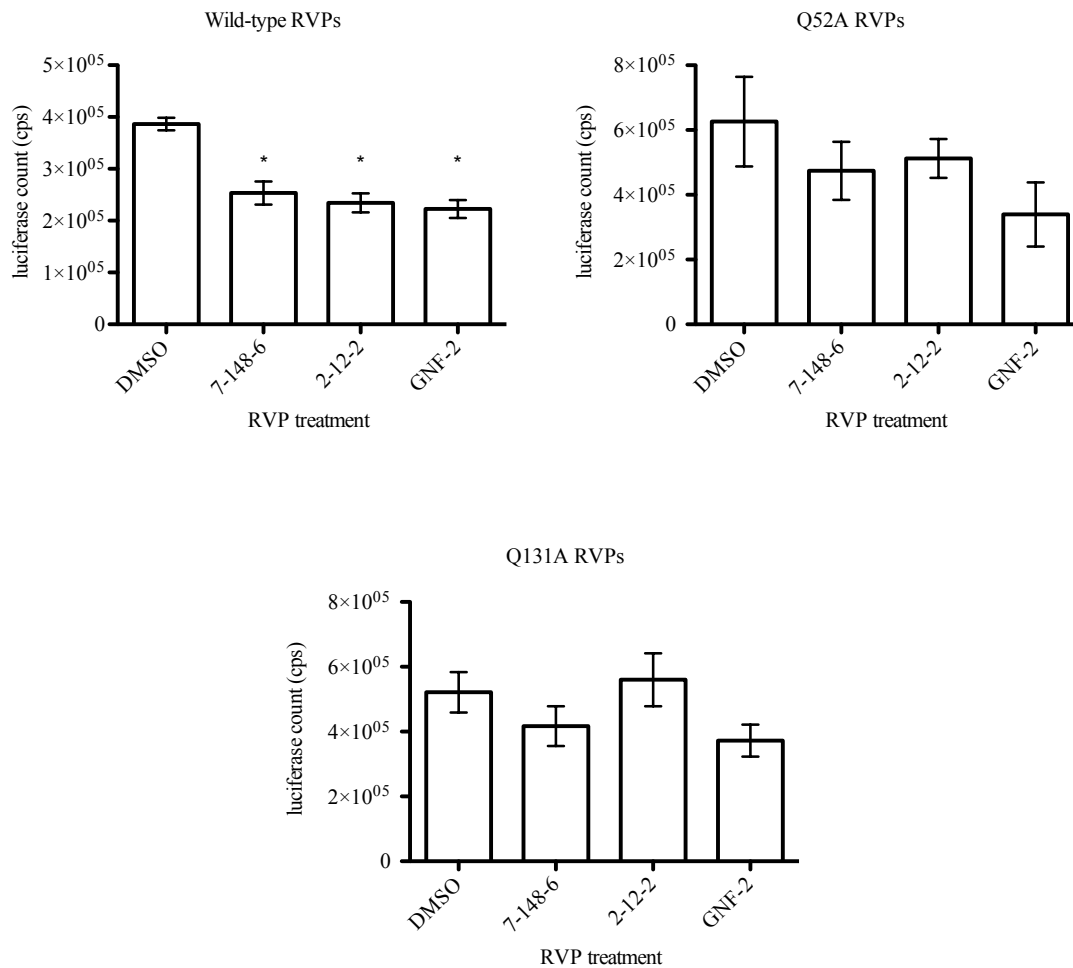
We next normalized to WNV genome copy number in the supernatant and treated RVPs with 7-148-6, 2-12-2, or GNF-2 prior to infecting target cells. Treatment of wild-type RVPs by each compound led to significant decreases of luciferase activity in target cells compared to a DMSO-treated control (Figure 4-12). In contrast, we found no significant decrease in either E(Q52A) or E(Q131A) RVPs when treated with any of these compounds. This suggests that these individual mutations may decrease sensitivity of the RVPs to compound inhibition.

## **DISCUSSION**

This chapter describes our investigation of effects of point mutations in the DENV E protein on viral entry and sensitivity to a subset of our small molecule entry inhibitors. Passaging of DENV2 in the presence of small molecules led to outgrowth of



**Figure 4-11. DENV envelope protein mutations affect virus particle production and entry in a single-cycle infection assay.** (A) RVPs in producer cell supernatant were quantified by performing qRT-PCR against the WNV replicon. Each bar represents the mean of three replicates with error bars showing standard deviation. (B) After quantification, RVPs were diluted to normalize for WNV copy number and BHK-21 cells were infected. Sixteen hours post-infection, cells were lysed and luciferase signal was measured. Each bar represents the mean of four replicates with error bars showing standard deviation. \*p value <.0.05



**Figure 4-12. E(Q52A) and E(Q131A) may confer resistance to small molecule entry inhibitors.** RVPs containing wild-type E, E(Q52A), or E(Q131A) were diluted to normalize for genome copy and pre-treated with the indicated compounds at their previously calculated EC<sub>90</sub> values against DENV2 (MOI of 1) before addition to cells. Cells were lysed 16 hours post-infection and luciferase signal was measured. Each bar represents the mean of four replicates with error bars showing standard deviation.

\*p value < 0.01

virus against only one out of ten small molecule, 7-148-6, suggesting that DENV does not easily escape the presence of the compounds. Consistent with this hypothesis are the results of our small alanine mutagenesis screen, in which eight of eleven mutations in the domain I/II interface of E drastically reduced viral particle production or entry.

Sequencing of DENV2 passaged in the presence of 7-148-6 identified two mutations that caused changes in the E and M proteins, the structural proteins on the virion surface. Characterization of the E(M196V) and M(H28Y) mutations in a single-cycle reporter virus assay suggests that these changes impact viral particle entry. E(M196V) enhanced entry of reporter virus particles and caused insensitivity to the compound 7-148-6, against which it was selected. M(H28Y) may also confer some degree of resistance to 7-148-6, although the marked decrease in entry of these virus particles makes this difficult to interpret. Despite the full recovery of entry signal in E(M196V) RVPs after treatment with 7-148-6, it is probable that this single E protein residue does not solely dictate compound sensitivity. This is due to the fact that the methionine at position 196 is conserved across all DENV serotypes, yet 7-148-6 did not inhibit DENV3 or DENV4, suggesting that other amino acids play a factor in the antiviral activity of 7-148-6.

We showed that compound 7-148-6 likely has a viral target, is active during DENV entry, and blocks DENV2 fusion *in vitro*. The results with E(M196V) RVPs are consistent with the hypothesis that 7-148-6's target is the E protein on the surface of the virion. At this point, it is unclear how the E(M196V) change causes resistance to 7-148-6. While escape mutations on proteins have traditionally been useful in inferring the binding sites of antivirals on their respective targets, it is unclear if the M196V change inhibits

binding of the compound directly or if it allows for escape by altering how the E protein mediates fusion or undergoes the conformational rearrangements that occur during fusion.

The E(M196V) change could impact fusion in a number of ways. Residue 196 is located at the base of the BOG pocket, in a position that may make contact with the lipid membrane of the virion; it is possible that the shorter side chain of valine at this position allows for easier movement of domain II away from the lipid membrane. Another possible hypothesis to explain enhanced virus entry or compound resistance is a mutation at this position could affect the formation or stability of the E trimer, as the pseudo-alpha helix (residues 192-196) is predicted to make intra-trimer contacts in the final post-fusion E structure. This hypothesis could also explain why the M196A change resulted in reduced entry of RVPs, as the smaller side chain of alanine could have a detrimental effect on trimer formation or stability. Alternatively, it is possible that the M196V change directly affects the ability of 7-148-6 to bind to the E protein. Our laboratory is currently performing studies with recombinant dengue envelope protein to test this possibility.

The other identified change in the structural proteins of DENV was a histidine to tyrosine change located in the ectodomain of the M protein, a mutation that significantly reduced RVP entry. On its own, it is unclear how this change affects entry or could confer resistance to 7-148-6. Little is known about what role, if any, the M protein plays in DENV infectivity after the pr peptide is cleaved (Mukhopadhyay et al, 2005). One study showed that, post-cleavage, the ectodomain of M rests partially buried in the virus lipid envelope underneath the E protein homodimer (Kuhn et al., 2002). It is possible that M makes contacts with E and could influence movement of domain II away from the viral membrane. It is also possible that M(H28Y) appeared in passaged DENV2 as a



compensatory mutation for E(M196V). One way to test this hypothesis would be to examine entry and compound sensitivity of RVPs that contain both E(M196V) and M(H28Y), experiments that we are currently performing.

The alanine mutagenesis screen of the BOG pocket on the E protein was performed to examine the effects of mutations in this region on viral entry. The majority of mutations we introduced appeared to affect viral particle production and/or entry, suggesting that changes in this region may have a fitness cost for the virus. Unfortunately, due to our inability to monitor the presence of E protein release into producer cell supernatant, we cannot rule out the possibility that some point mutations that appear to affect viral particle production or release may, in fact, have interfered with earlier steps of the DENV life cycle such as production of E or coating of immature viral particles

The contrasting results of E(M196V) and E(M196A) in the RVP system suggest that specific substitutions may dictate the effect of mutations in E on viral entry, making it difficult to draw conclusions from our limited mutagenesis screen about the contribution of specific residues. In order to better understand potential mechanisms of resistance and the impact of resistance mutations on fitness, additional mutagenesis experiments should be performed targeting residues in this region that are not conserved or that have not been implicated in altering the pH of viral fusion.

A recently published study reported site-directed mutagenesis of several residues around the BOG pocket in authentic DENV and, similar to our results, found that many of their mutations had effects on DENV entry or viability (Butrapet et al., 2011). Two mutations in their study, F193A and F279A, were identical to mutations that we examined in the RVP system. The E(F193A) mutant virus was found to have lower

replication efficiency while the E(F279A) mutant virus exhibited no change in the pH threshold of fusion or in viral replication, although this mutant virus was found after several passages to have a mixed population of alanine and leucine at position 279. In the RVP system, we found that both of these mutations inhibited RVP production and would be expected to cause reduced viral replication in the context of the authentic virus. However, since the RVP system permits only single-cycle infection, it does not allow for compensatory or reversion mutants, and this may provide a possible explanation for apparent discrepancies between our study and the previous results using authentic virus.

Interestingly, Butrapet et al. (2011) also mutated Q52, although to an arginine instead of alanine. In RVPs, we found that Q52A caused a significant inhibition of viral particle production, but when normalized for WNV genome copy, significantly increased RVP entry (Figure 4-11). In authentic virus, Butrapet et al found that Q52R caused increased cytopathic effect upon passaging in cells and possibly a higher pH threshold for fusion. An explanation consistent with both sets of results is that mutations at residue 52 lead to enhancement of DENV fusion.

We tested E(Q52A) and E(Q131A) against a subset of our small molecule entry inhibitors and found that both changes conferred resistance to all three compounds compared to wild-type RVPs. As with E(M196V), we do not currently know how these changes confer resistance. Residues 52 and 131 are located close to the mouth of the BOG pocket, while 196 sits at the very base (Figure 4-9), making it unlikely that all three changes inhibit binding of small molecules. A more likely explanation is that one or more of these changes alter DENV fusion as a means of escaping small molecule inhibition.

In the work presented here, we have begun to explore how changes to the DENV E protein influence both the entry of virus particles as well as susceptibility to small molecule entry inhibitors. We identified a point mutation in the envelope protein, E(M196V) that rendered a single-cycle reporter virus insensitive to the presence of the compound it was raised against. This is the first recorded instance of a potential resistance mutation to a DENV small molecule entry inhibitor. However, further work should be done to characterize this resistance mutation as well as explore the mechanism of resistance. The mutation should be introduced back into authentic dengue virions, which would allow for biochemical studies to examine DENV fusion in the presence or absence of 7-148-6. In addition, we show that specific substitutions in the hinge region of E can impact viral particle production or entry. These results provide promising leads to learn more about the molecular interaction of our DENV small molecule entry inhibitors with the E protein as well as to discover more about the functional contribution of individual amino acids in the E protein to DENV fusion.

## **METHODS AND MATERIALS**

### **DENV passaging**

Initially, DENV2 (MOI 0.1) was incubated with selected compounds (see text) for 45 minutes at 37°C before addition to  $10^6$  Vero cells in a T25 flask. After a one hour infection, 5 ml medium was added (2% FBS in DMEM) containing compound or DMSO. Infections proceeded for four days, at which point supernatant was harvested and spun briefly to remove cell debris. Five hundred  $\mu$ L of supernatant was used to infect a fresh T25 of Vero for one hour, at which point 4.5 ml of medium containing compound was added. Viral titer of each passage was determined by plaque-forming assay. The first passaging with compounds maintained 20  $\mu$ M of each compound over eight passages. The second passaging started at 2.5  $\mu$ M and increased by 2.5  $\mu$ M each passage until 20  $\mu$ M was reached, which was kept constant for two passages.

### **Order of addition experiments**

DENV2 (NGC strain) was used to infect BHK-21 cells at an MOI of 1 for all experiments. Compound was used at 10  $\mu$ M. Virus was pre-incubated in 100  $\mu$ L of medium containing compound at 37°C. For co-infection, 10  $\mu$ M of compound or DMSO was added to virus inoculum directly before addition to cells. After an initial one hour infection, cells were washed twice with 1X PBS to remove unbound compound and virus, and 1 mL cell medium (MEM-alpha with 2% FBS) was added. For post-treatment of cells, compound was added to medium for 1 hour post-infection at 10  $\mu$ M, then removed and cells were washed twice and medium was replaced. Twenty-four hours after initial infection, supernatant was collected and viral titer was determined by plaque-forming assay.

### **Removal of unbound compounds by gel filtration column**

DENV2 (NGC strain) inocula (MOI 2) was pre-incubated in 100  $\mu$ L with 10  $\mu$ M of compound or DMSO for 45 minutes at 37°C, then placed over a Sephadex G50 spin column and spun for 2 minutes at 2000 rpm at room temperature. Eluant was used to infect cells for 1 hour, after which cells were washed twice with 1X PBS to remove unbound compound and virus, and 1 mL cell medium (MEM-alpha with 2% FBS) was added. Twenty-four hours after initial infection, supernatant was collected, and viral titer was determined by plaque-forming assay.

### **DENV attachment to cells**

DENV2 (NGC strain) was incubated with either 5  $\mu$ M of 2-12-2 or 8-24-3, 20  $\mu$ g/ml of heparin, or DMSO for 45 minutes at 37°C, then added to BHK cells (MOI 1) at 4°C for one hour with constant rocking. Cells were washed three times with cold 1X PBS to remove unbound virus or compound. For quantitation of viral genomes bound, total RNA was extracted using Trizol according to manufacturer's instructions and qRT-PCR was performed. For viral yield determination, one mL of medium was added (MEM-alpha with 2% FBS), and culture supernatant was harvested 24 hours later. To determine viral titer in supernatant, plaque-forming assays were performed.

### **Quantification of DENV genomes**

Quantification of DENV genomes was done on a MyIQ Cyclor (Biorad). After RNA extraction and quantification, 5  $\mu$ L of RNA was used to make cDNA with the iScript kit (Biorad) according to manufacturer's instructions. The resulting cDNA was digested by RNaseH for 1 hour at 37°C. Quantification by rt-PCR was done on each sample in

triplicate (25  $\mu$ L each) using SybrGreen Supermix (Biorad) according to manufacturer's instructions. Primers used targeted the DENV C gene.

DV2Cfor 5'-AATATGCTGAAACGCGAGAGA-3'

DV2Crev 5'-GGGATTGTTAGGAAACGAAGG-3'

### **Liposomes**

Liposomes were made with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) (Avanti Polar Lipids cat # 850457 and 850757, respectively), and cholesterol (Sigma-Aldrich cat#C3045) at 1:1:2 molar ratio in TAN buffer (20mM triethanolamine, 100mM NaCl, pH 8.0). Lipids were dried down and resuspended thoroughly by vortexing and sonication. Liposomes were prepared by extrusion through a 0.2 micron filter after five freeze/thaw cycles. For trypsin-containing liposomes, 10 mg of trypsin was added to 1 mL of lipids (3 mg each) after the third freeze/thaw cycle, prior to extrusion. Liposomes were separated from unincorporated trypsin by size-exclusion chromatography using a Superdex 200 column on an Akta fast performance liquid chromatography (FPLC) system. Trypsin-containing liposomes were used within 24 hours.

### **Capsid protection assay**

Purified dengue 2 virus (300 ng) was incubated with various concentrations of compounds (5-40  $\mu$ M) for 45 minutes at 37°C in TAN buffer (pH 8.0) prior to addition of liposomes (7.5  $\mu$ L of peak fraction from column) for ten minutes. After incubation with liposomes, 7.5  $\mu$ L 1M sodium acetate (pH 5.0) was added to drop pH to 5.5 for ten minutes. Samples were back-neutralized with 15  $\mu$ L 1M TEA (unbuffered) and incubated for forty-five minutes at 37°C to allow trypsin digestion (total volume: 100  $\mu$ L). SDS

sample buffer (20  $\mu$ L) was added, and samples were boiled for 20 minutes before being separated by SDS-PAGE on a 12% gel. Proteins were transferred to a nitrocellulose membrane using a semi-dry transfer. 4G2 antibody, grown in house from ATCC cell line HB-112, was used for detection of DENV envelope (dilution varies by lot), and the neat supernatant of hybridoma cell line 6F3.1, kindly provided by Dr. John Aaskov, was used to detect capsid protein.

### **Production of recombinant virus particles**

Plasmids for WNV replicon and DENV structural proteins were the kind gift of Dr. Ted Pierson. RVP production was performed according to protocols received from his lab (Ansarah-Sobrinho et al, 2008). 293T-LX cells were transfected with plasmids using Lipofectamine 2000 according to manufacturer's instructions at a ratio of 3:1 (DENV CprME:WNV replicon). Medium was changed 4 hours post-transfection and supernatant was collected at 25 hours post-transfection. Supernatant was spun for 5 minutes at 1200 rpm to remove cell debris and was put through a 0.2 micron filter before freezing. Viral RNA was extracted using the QIAamp Viral RNA kit according to manufacturer's instructions. RNA was transcribed to cDNA using iScript cDNA kit (Biorad) according to manufacturer's instructions and qPCR was performed using primers against the WNV replicon to quantify genome copies.

### **Quantification of WNV replicon**

Quantification of WNV replicons in RVP supernatant was done on a MyIQ Cyclor (Biorad). RVP RNA was extracted using the QIAamp Viral RNA Mini kit from Qiagen (Cat#52906) and suspended in a final volume of 60  $\mu$ L, 2  $\mu$ L of which was used to make cDNA with the iScript kit (Biorad) according to manufacturer's instructions. The

resulting cDNA was digested by RNaseH for 1 hour at 37°C. Quantification by rt-PCR was done on each sample in triplicate (25 µL/well) using SybrGreen Supermix (Biorad) according to manufacturer's instructions. Sequences of primers against the WNV 3' UTR replicon are listed below.

WNVfwd: 5'-AGAGTGCAGTCTGCGATAGTGC-3'

WNVrev: 5'-CCCTTCTCCCTGGTTAACA-3'

### **Site-directed mutagenesis of DENV CprME plasmid**

Site-directed mutagenesis of the DENV CprME plasmid was performed using the Stratagene Quikchange II kit according to manufacturer's instructions (Cat#200518). Primers to introduce all mutations were designed using the Quikchange Primer Design website (sequences listed below). XL10 Gold competent cells were transformed with mutated plasmids after digestion and grown at 30°C for 2 days. Selected colonies were grown in superbroth at 30°C for 24 hours, then plasmids were isolated and sequenced to confirm the presence of mutations.

M(H28Y): *c1652t* 5'-cagaaggggcctggaaatatgtccagagaattga-3'; *c1652t\_antisense* 5'-tcaattctctggacatattccaggcccccttg-3'

E(M196V): *a238lg* 5'-gcctcgactcaatgaggtggtgtgtgcagatg-3'; *a238lg\_antisense* 5'-catctgcaacaacaccacctcattgaagtcgaggc-3'

E(Q52A): *c1949g\_a1950c* 5'-tgaactgataaaaacagaagccaaagcgctgccacccta-3';  
*c1949g\_a1950c\_antisense* 5'-taggggtggcaggcgcttggcttctgttttatcagttca-3'

E(V130A): *t2184c* 5'-gaacatggaaggaaaagttgcgcaaccagaaaacttggaat-3'; *t2184c\_antisense* 5'-attccaagtttctggttgcgcaacttttccttccatgttc-3'



E(Q131A): *c2186g\_a2187c* 5'-gaacatggaaggaaaaagttgtggcaccagaaaacttggaatacacc-3';  
*c2186g\_a2187c\_antisense* 5'-ggtgtattccaagttttctggtgccacaacttttcttccatgttc-3'

E(D192A): *a2370c* 5'-ccaagaacgggcctcgcttcaatgagatggtg-3'; *a2370c\_antisense* 5'-  
caccatctcattgaaggcgaggccccgttcttgg-3'

E(F193A): *t2372g\_t2373c* 5'-ccaagaacgggcctcgacgccaatgagatggtgtgtt-3';  
*t2372g\_t2373c\_antisense* 5'-aacaacaccatctcattggcgtcgaggccccgttcttgg-3'

E(N194A): *a2375g\_a2376c* 5'-gaacgggcctcgacttcgctgagatggtgttgc-3';  
*a2375g\_a2376c\_antisense* 5'-gcaacaacaccatctcagcgaagtcgaggccccgttc-3'

E(M196A): *a2381g\_t2382c* 5'-ggcctcgacttcaatgaggcgggtgtgtgcagatgga-3';  
*a2381g\_t2382c\_antisense* 5'-tccatctgcaacaacaccgcctcattgaagtcgaggcc-3'

E(G275A): *g2619c* 5'-agaaatccaaatgtcatcagcaaacttactcttcacaggac-3'; *g2619c\_antisense* 5'-  
gtcctgtgaagagtaagtttgctgatgacatttgattct-3'

E(L277A): *t2624g\_t2625c* 5'-aatccaaatgtcatcaggaaacgcactcttcacaggacatctcaag-3';  
*t2624g\_t2625c\_antisense* 5'-cttgagatgtcctgtgaagagtgcgtttcctgatgacatttgatt-3'

E(L278A): *c2627g\_t2628c* 5'-cagaaatccaaatgtcatcaggaaacttagccttcacaggacatctc-3';  
*c2627g\_t2628c\_antisense* 5'-gagatgtcctgtgaaggctaagtttctgatgacatttgattctg-3'

E(F279A): *t2630g\_t2631c* 5'-gtcatcaggaaacttactcgccacaggacatctcaagtgc-3';  
*t2630g\_t2631c\_antisense* 5'-gcacttgagatgtcctgtggcgagtaagtttctgatgac-3'

### **RVP infection of cells**

RVPs were used to infect BHK-21 cells in 48-well plates. RVPs were diluted to  $10^5$  genomes/mL and incubated with small molecules at their respective EC<sub>90</sub> values (as determined in cellular entry assays) for 45 minutes at 37°C. This mixture was added to cells for a one hour initial infection (37°C), after which cells were washed twice with 1X

PBS. All infections were done in quadruplicate. Medium was added (2% FBS in MEM-alpha) and cells were incubated at 37°C for 16 hours. At 16 hours post-infection, cells were washed once before lysis and lysate was moved to -20°C until use. Luciferase signal was determined with a Renilla luciferase activity kit (Promega Cat#E2820).

## REFERENCES:

- Ansarah-Sobrinho, C., Nelson, S., Jost, C.A., Whitehead, S.S., and Pierson, T.C. (2008). Temperature-dependent production of pseudoinfectious dengue reporter virus particles by complementation. *Virology* 381, 67-74.
- Beasley, D.W.C., and Aaskov, J.G. (2001). Epitopes on the Dengue 1 Virus Envelope Protein Recognized by Neutralizing IgM Monoclonal Antibodies. *Virology* 279, 447-458.
- Butrapet, S., Childers, T., Moss, K.J., Erb, S.M., Luy, B.E., Calvert, A.E., Blair, C.D., Roehrig, J.T., and Huang, C.Y.H. (2011). Amino acid changes within the E protein hinge region that affect dengue virus type 2 infectivity and fusion. *Virology* 413, 118-127.
- Gollins, S.W., and Porterfield, J.S. (1986). The Uncoating and Infectivity of the Flavivirus West Nile on Interaction with Cells: Effects of pH and Ammonium Chloride. *Journal of General Virology* 67, 1941-1950.
- Kampmann, T., Yennamalli, R., Campbell, P., Stoermer, M.J., Fairlie, D.P., Kobe, B., and Young, P.R. (2009). In silico screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses. *Antiviral Research* 84, 234-241.
- Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., *et al.* (2002). Structure of Dengue Virus: Implications for Flavivirus Organization, Maturation, and Fusion. *Cell* 108, 717-725.
- Lee, E., Weir, R.C., and Dalgarno, L. (1997). Changes in the Dengue Virus Major Envelope Protein on Passaging and Their Localization on the Three-Dimensional Structure of the Protein. *Virology* 232, 281-290.
- Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America* 100, 6986-6991.
- Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature* 427, 313-319.
- Mukhopadhyay, S., Kuhn, R.J., and Rossmann, M.G. (2005). A structural perspective of the flavivirus life cycle. *Nat Rev Micro* 3, 13-22.
- Poh, M.K., Yip, A., Zhang, S., Priestle, J.P., Ma, N.L., Smit, J.M., Wilschut, J., Shi, P.-Y., Wenk, M.R., and Schul, W. (2009). A small molecule fusion inhibitor of dengue virus. *Antiviral Research* 84, 260-266.

Schmidt, A.G., Lee, K., Yang, P.L., and Harrison, S.C. (2012). Small-Molecule Inhibitors of Dengue-Virus Entry. *PLoS Pathog* 8, e1002627.

Wang, P.-G., Kudelko, M., Lo, J., Siu, L.Y.L., Kwok, K.T.H., Sachse, M., Nicholls, J.M., Bruzzone, R., Altmeyer, R.M., and Nal, B.a. (2009a). Efficient Assembly and Secretion of Recombinant Subviral Particles of the Four Dengue Serotypes Using Native prM and E Proteins. *PLoS ONE* 4, e8325.

Wang, Q.-Y., Patel, S.J., Vangrevelinghe, E., Xu, H.Y., Rao, R., Jaber, D., Schul, W., Gu, F., Heudi, O., Ma, N.L., *et al.* (2009b). A Small Molecule Dengue Virus Entry Inhibitor. *Antimicrob Agents Chemother*, AAC.01148-01108.

WHO (2012). Dengue and severe dengue.

Zhang, W., Chipman, P.R., Corver, J., Johnson, P.R., Zhang, Y., Mukhopadhyay, S., Baker, T.S., Strauss, J.H., Rossmann, M.G., and Kuhn, R.J. (2003a). Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. *Nature Structural & Molecular Biology* 10, 6.

Zhang, Y., Corver, J., Chipman, P.R., Zhang, W., Pletnev, S.V., Sedlak, D., Baker, T.S., Strauss, J.H., Kuhn, R.J., and Rossmann, M.G. (2003b). Structures of immature flavivirus particles. *EMBO* 22, 10.

## **Chapter 5:** Discussion

## SUMMARY

The project covered in this dissertation stems from an unexpected observation: namely, that DENV yield was lowered when initial virus inoculum was pre-incubated with GNF-2, a previously identified small molecule inhibitor of intracellular Abl kinases. This discovery serves as a reminder of the importance of exploring the possibility of off-target effects for many scientific approaches, from RNAi to small molecules to peptides.

Chapter 2 discusses our initial observation of an unexpected drop in DENV yield when virus inoculum is pre-incubated with a small molecule inhibitor of cellular Abl kinases, GNF-2. Further experiments revealed that GNF-2 interacts with purified DENV virions as well as recombinant envelope protein dimer, the pre-fusion form of the envelope found on the surface of mature virions. Other small molecule inhibitors have previously been reported to interact with dengue, but these were discovered through high-throughput and *in silico* screens designed to identify compounds that bind directly to the envelope protein of the virion (Kampmann et al., 2009; Poh et al., 2009; Schmidt et al., 2012; Wang et al., 2009; Zhou et al., 2008). In contrast, GNF-2 is well established as an inhibitor of Abl kinases that derives its high degree of selectivity by binding in the myristate-binding pocket of the kinase and is inactive against over 450 other cellular kinases (Adrian et al., 2006; Zhang et al., 2010). Our discovery that GNF-2 inhibits DENV at two separate points in the viral life cycle raises the intriguing possibility of designing small molecule inhibitors that block viruses via two targets at separate points during the viral life cycle.

As a way to separate GNF-2's two anti-DENV activities – one mediated by intracellular Abl kinase and one mediated by interactions with the dengue virion – we

performed a structure-activity relationship study in collaboration with Dr. Nathanael Gray's laboratory. This led to the identification of twenty-three small molecules that inhibited DENV infectivity with equivalent or improved activity relative to GNF-2. All twenty-three molecules showed no inhibition of Abl kinases in cellular assays. In Chapter 3, we selected two of these compounds for further characterization. We found that DENV yield was not inhibited if virus is pre-incubated with compound at 4°C, suggesting dynamic motion of the virion surface may be necessary to allow binding of compounds. When we examined specific steps of the DENV entry process, we found that the compounds did not inhibit attachment of virions to cells, but did prevent completion of viral fusion *in vitro*. These results are consistent with our current hypothesis that the small molecule inhibitors interfere with the significant structural changes that the DENV E protein must undergo during viral entry, although additional experiments are needed to provide support for and refine this hypothesis. The SAR study and mechanistic work that I have presented add to the growing body of identified DENV entry inhibitors that prevent viral fusion.

In Chapter 4, we utilized a classical virology approach to learn more about the mechanism of action of our DENV entry inhibitors. We passaged virus in the presence of ten compounds, and observed outgrowth of an insensitive virus against only one, 7-148-6. Sequencing of the structural genes of this virus quasispecies revealed two point mutations; one each in the E and M proteins. Using a single-cycle reporter virus assay, we found that treatment of wild-type virus particles with 7-148-6 reduced viral entry, but no drop was observed with virus particles containing the E(M19V) or M(H28Y) mutation, suggesting that both changes may play a role in the observed resistance of the

authentic virus. We then performed an alanine scanning study focused on the region surrounding the E(M196V) mutation and, using the single-cycle reporter virus, found that the majority of point mutations in this area were not well-tolerated, decreasing either viral particle production or entry. Two mutations, E(Q52A) and E(Q131A), showed efficient virus entry and were subsequently tested against a subset of our small molecule entry inhibitors. Both mutations exhibited reduced sensitivity to all three small molecules tested when compared to the compounds' effect on wild-type virus particles.

Interestingly, the E(M196V), E(Q52A), and E(Q131A) reporter viruses all had higher infectivity than wild type viruses, as reflected by luciferase signal per genome copy. This possible enhancement of viral entry may explain the reduced sensitivity to compounds. Alternatively, reduced sensitivity to the entry inhibitors may indicate that the E protein mutations interfere with the interaction of compounds with E. Additional experiments that elucidate not only the interactions mediating binding of the inhibitors to wild-type and mutant E proteins but also the mechanism(s) of action of our entry inhibitors are needed to distinguish between these two potential mechanisms of resistance, which are not mutually exclusive. Our current results suggest that point mutations in the E domain I/II interface may affect sensitivity to entry inhibitors and supports previous work that individual residues in this region can impact DENV entry.

## **DISCUSSION AND FUTURE DIRECTIONS**

The need for DENV therapeutics is undeniable; cases of DENV fever and severe fever have increased exponentially over the past four decades and there are still no specific vaccines or treatments available (WHO, 2012). However, the search for effective



antivirals is hampered by a lack of understanding of many of the basic viral processes that DENV undergoes as well as what cellular proteins are critical for viral infection. Until recently, the virus was endemic largely in countries that did not have resources to devote to basic research of the virus (WHO, 2012). The reason for the initial screen that led to this dissertation and identified GNF-2 as an inhibitor of DENV was to identify cellular kinases critical for DENV infection (Chu and Yang, 2007). While a long-term goal of our work is to contribute to the search for effective antivirals, a more immediate goal is to investigate the DENV life cycle to identify molecules and processes that may be pharmacologically targeted to inhibit the virus.

### **DENV entry as an antiviral target**

Dengue virus encodes only a small number of viral proteins, thus making it highly dependent upon host cells to successfully complete a life cycle. In order to take advantage of the host cell machinery, DENV must first enter cells to gain access to the host cytoplasm, where viral transcription and replication occur. DENV is taken up into cells via clathrin-mediated endocytosis (Acosta et al., 2008; van der Schaar et al., 2008). It is then trafficked to cellular endosomes, where exposure to the low pH triggers viral fusion with the cellular membrane (Stiasny and Heinz, 2006; van der Schaar et al., 2008). Following viral protein production and genome replication, nascent virions are assembled, packaged, and undergo maturation during exocytosis prior to the start of a new round of infection.

Each step in the viral life cycle presents a potential point for therapeutic intervention, but viral entry may be considered particularly appealing for several reasons. DENV entry is a discrete process and, relative to other viral processes such as replication

or assembly, may be of limited scope in terms of viral and cellular proteins involved. In addition, the initial interaction between virus and cell occurs on the cell exterior, allowing the virion to be targeted by non-cell permeable inhibitors or even antibodies. Finally, viral entry occurs prior to genome replication, thereby decreasing the opportunity for viral resistance to emerge.

### **Limitations of GNF-2 and current inhibitors as potential antivirals**

Importantly, our discovery that GNF-2 and the derivatives identified in the structure-activity relationship (SAR) study (Appendix A) inhibit DENV entry does not automatically qualify them as promising DENV therapeutics. One limitation of the compounds we identified is that none of them potently inhibited all four DENV serotypes. During a DENV outbreak, it can initially be unclear which serotype or serotypes are circulating, and thus it would be desirable for therapeutics to be active against all four serotypes. It is important to note that our efforts in the SAR study were directed at optimizing inhibition of DENV2 and only tested against other serotypes after potency of DENV2 inhibition was determined. Even with the focus on DENV2, we were able to identify five compounds that had measurable EC<sub>90</sub> values against all four serotypes, suggesting that identification of pan-serotype inhibitors may be possible. A second limitation to the direct development of inhibitors such as 2-12-2, 7-148-6, and 8-24-3 as antivirals is that their potency may be too low (EC<sub>90</sub> values in the low single-digit micromolar) to be useful antivirals *in vivo*. While this could be unequivocally established via testing in an available animal model of DENV infection (Zompi and Harris, 2012), it is likely that additional optimization of inhibitor potency would be necessary to achieve therapeutically beneficial inhibition of DENV *in vivo*.

We are not the first group to identify small molecule inhibitors of DENV entry. GNF-2 and the other twenty-one small molecules identified in this study add to the growing body of DENV entry inhibitors that purportedly act by inhibiting viral fusion (Table 1-1). Our compounds are not as potent as some other small molecules such as NITD6 (reported EC<sub>50</sub> value 119 nM) (Wang et al., 2009), 3-110-22 (reported IC<sub>90</sub> value 790 nM) (Schmidt et al., 2012), or peptide inhibitors based on the stem region of E (reported EC<sub>90</sub> value 1  $\mu$ M (Schmidt et al., 2010), but they do exhibit potencies comparable to or exceeding leading compounds from several other studies, including a peptide inhibitor with a reported EC<sub>50</sub> value of 10  $\mu$ M (Hrobowski et al., 2005), small molecule A5 (reported EC<sub>50</sub> value 4  $\mu$ M) (Yennamalli et al., 2009), small molecule P02 (reported EC<sub>50</sub> 13  $\mu$ M) (Zhou et al., 2008) and small molecule NITD448 (reported EC<sub>50</sub> value 9.8  $\mu$ M) (Poh et al., 2009). It is worth noting, however, that we calculated the EC<sub>90</sub> values of our compounds, while most other studies calculated EC<sub>50</sub> values of compounds. Consequently, direct comparisons of anti-DENV potency must await side-by-side comparisons of our compounds with reference compounds from prior studies, as we have done with NITD6 (Chapter 2).

In addition, we note that in our SAR optimization, we utilized a relatively low-throughput assay measuring differences in DENV yield. In the future, utilization of a biochemical assay based on fluorescence polarization measurements to monitor interaction of compounds with the soluble pre-fusion E dimer or cellular assays utilizing the single-cycle reporter virus system may facilitate broader screening efforts and enable more sensitive and quantitative detection of the antiviral activities of the GNF-2-inspired entry inhibitors described in this study.

### **Probing mechanism(s) of action using biochemical assays and mutagenesis**

As with most scientific research, the experiments performed in this dissertation raise a number of further questions. While our biochemical *in vitro* experiments reveal that the two tested compounds, 2-12-2 and 8-24-3, prevent the completion of DENV fusion, we do not know exactly at what step this block occurs. The compounds could prevent lipid mixing between the viral and cellular membranes or they may block at a another step, such as the fold-back of the E domain III or final formation of the fusion pore (Figure 1-3). It is possible to test whether lipid mixing has occurred *in vitro* using pyrene-labeled virus and liposomes as a target membrane (Schmidt et al., 2010), although our attempts at this delicate assay with DENV did not yield sufficient signal to confidently detect fusion with untreated wild-type virus. However, if this assay were optimized and had increased sensitivity, it may be a viable approach to differentiate among these possible inhibitory mechanisms.

Another, broader, question about mechanism is whether GNF-2 and other compounds that were not tested in Chapters 3 or 4 inhibit the same step of DENV entry/fusion as 2-12-2, 8-24-3, and 7-148-6. It remains to be determined whether all of the compounds identified in our SAR study bind to the same site on E or inhibit DENV entry via the same molecule mechanism. It is therefore of interest to use our *in vitro* biochemical fusion assays to test a broader panel of compounds as well as test the above compounds against other DENV serotypes. Likewise, efforts to identify the binding site(s) of these compounds on the E structure via high-resolution structure determination are of considerable interest.

In the meantime, the results from our mutagenesis studies constitute an important step towards examining the potential mechanism of action of our DENV entry inhibitors while also serving as a broader study of the fusion of this region of the E protein in DENV entry. The E protein DI/DII interface has been of great interest to the flavivirus field since 2003, when the possibility of inhibiting DENV entry by targeting this region with a small molecule was first proposed (Modis et al., 2003). However, little experimental work has been done to explore this region outside of using it to model or test small molecule inhibitors. One recent study (Butrapet et al., 2011) performed a mutational analysis in this region of the protein using reverse genetics and found that in Vero cells, eight out of 15 selected residues were either lethal or unstable (i.e., did not retain the engineered mutation or contained purported compensatory mutations in other locations). These data, along with the RVP results in our studies demonstrating that point mutations in the domain I/II interface potentially affect expression, release, and/or entry of virus particles, suggest that changes in this area may have wide-ranging effects in the DENV life cycle. Further cellular and biochemical experiments are needed to characterize the phenotypic block in the DENV life cycle caused by mutations in this region of E. Elucidation of the relationship between structure and function of this region of the E protein should aid in rational design of either entry inhibitors or antivirals targeted to other steps of DENV infection.

While our RVP data suggest that the E(M196V) mutation potentially enhances viral entry, there are still many unanswered questions about this mutation, both within and outside the context of our small molecule inhibitors. Our group is currently working to determine whether the E(M196V) mutation alters the ability of recombinant envelope

protein to associate or interact with GNF-2 and other small molecules. This work will help clarify how this mutation confers resistance to the small molecule inhibitors; if the compound has reduced affinity for the E(M196V) protein, this suggests that resistance is conferred by prevention of compound binding. Otherwise, E(M196V) may confer resistance by causing E to become hyperfusogenic (Biering et al., 2012; Higgs and Gould, 1991) thus allowing the virus to complete fusion despite the presence of the compounds. E(M196V) could also alter the optimal pH of fusion, an event that has previously been suggested to occur when changes are made in the domain I/II interface (Beasley and Aaskov, 2001; Lee et al., 1997), and which could be tested by using virus containing this change in the *in vitro* capsid protection assay, where the pH drop can be tightly controlled. These two models are both consistent with our existing data and are not mutually exclusive.

In addition, we are studying the E(M196V) and M(H28Y) mutations in the context of full infectious virus by isolating plaque-purified DENV containing either or both mutations. Efforts are underway to measure the effect of these mutations on viral infectivity in the presence and absence of small molecule inhibitors and the behavior of these mutant viruses in biochemical fusion assays. We hope these results will validate the conclusions drawn from our single-cycle reporter virus studies. Likewise, independent introduction of these mutations to a molecular clone of DENV via reverse genetics will ensure that the phenotypes observed are the consequence of the introduced mutations and not due to previously undetected mutations in the DENV genome.

The work presented in this dissertation provides functional confirmation of the importance of the DI/DII interface of the E protein in DENV entry and adds to the growing body of knowledge of small molecule inhibitors of DENV entry. Although not the focus of my dissertation, GNF-2's inhibition of DENV via two separate targets via two independent mechanisms of action is also worthy of note. The concept of using multi-targeted compounds to achieve a maximal therapeutic index is gaining traction in other areas of biomedicine, notably oncology (Dar et al., 2012). Our early work with GNF-2 provides conceptual support for the development of dual-action, dual-target small molecule inhibitors of DENV that may have higher barriers to viral resistance. We found that the compounds most likely target the E protein on the surface of the virion and block a late stage in the viral fusion cascade. In pursuing further details about how the compounds interact with the E protein, we found that single point mutation(s) in the envelope protein can confer resistance to the compounds. While we do not yet know the mechanism of resistance, our mutational screen of the domain I/II interface of E revealed that changes in this area may have drastic effects on not only entry of the virus, but also egress or release of virions. Somewhat ironically, while much of the work described in this dissertation has been focused towards development of small molecules that act via a single target in order to aid our efforts to establish E as a target mediating GNF-2's inhibition of DENV entry, the development of GNF-2 or GNF-2-inspired compounds as potential anti-DENV agents might instead aim at optimizing the activity against Abl and E simultaneously.

## REFERENCES:

- Acosta, E.G., Castilla, V., and Damonte, E.B. (2008). Functional entry of dengue virus into *Aedes albopictus* mosquito cells is dependent on clathrin-mediated endocytosis. *Journal of General Virology* 89, 474-484.
- Adrian, F.J., Ding, Q., Sim, T., Velentza, A., Sloan, C., Liu, Y., Zhang, G., Hur, W., Ding, S., Manley, P., *et al.* (2006). Allosteric inhibitors of Bcr-abl-dependent cell proliferation. *Nat Chem Biol* 2, 95-102.
- Beasley, D.W.C., and Aaskov, J.G. (2001). Epitopes on the Dengue 1 Virus Envelope Protein Recognized by Neutralizing IgM Monoclonal Antibodies. *Virology* 279, 447-458.
- Biering, S.B., Huang, A., Vu, A.T., Robinson, L.R., Bradel-Tretheway, B., Choi, E., Lee, B., and Aguilar, H.C. (2012). N-Glycans on the Nipah Virus Attachment Glycoprotein Modulate Fusion and Viral Entry as They Protect against Antibody Neutralization. *Journal of Virology* 86, 11991-12002.
- Butrapet, S., Childers, T., Moss, K.J., Erb, S.M., Luy, B.E., Calvert, A.E., Blair, C.D., Roehrig, J.T., and Huang, C.Y.H. (2011). Amino acid changes within the E protein hinge region that affect dengue virus type 2 infectivity and fusion. *Virology* 413, 118-127.
- Chu, J.J.H., and Yang, P.L. (2007). c-Src protein kinase inhibitors block assembly and maturation of dengue virus. *Proceedings of the National Academy of Sciences* 104, 3520-3525.
- Dar, A.C., Das, T.K., Shokat, K.M., and Cagan, R.L. (2012). Chemical genetic discovery of targets and anti-targets for cancer polypharmacology. *Nature* 486, 5.
- Higgs, S., and Gould, E. (1991). Difference in fusogenicity and mouse neurovirulence of Japanese encephalitis virus. *Archives of Virology* 119, 14.
- Hrobowski, Y.M., Garry, R.F., and Michael, S.F. (2005). Peptide inhibitors of dengue virus and West Nile virus infectivity. *Virology Journal* 2.
- Kampmann, T., Yennamalli, R., Campbell, P., Stoermer, M.J., Fairlie, D.P., Kobe, B., and Young, P.R. (2009). In silico screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses. *Antiviral Research* 84, 234-241.
- Lee, E., Weir, R.C., and Dalgarno, L. (1997). Changes in the Dengue Virus Major Envelope Protein on Passaging and Their Localization on the Three-Dimensional Structure of the Protein. *Virology* 232, 281-290.

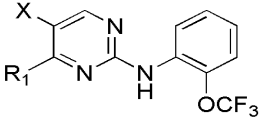




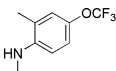
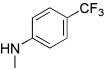
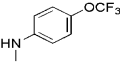
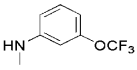


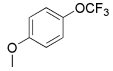
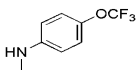


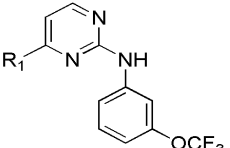

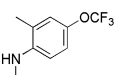
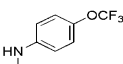
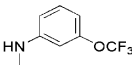


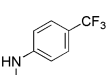
- Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America* 100, 6986-6991.
- Poh, M.K., Yip, A., Zhang, S., Priestle, J.P., Ma, N.L., Smit, J.M., Wilschut, J., Shi, P.-Y., Wenk, M.R., and Schul, W. (2009). A small molecule fusion inhibitor of dengue virus. *Antiviral Research* 84, 260-266.
- Schmidt, A.G., Lee, K., Yang, P.L., and Harrison, S.C. (2012). Small-Molecule Inhibitors of Dengue-Virus Entry. *PLoS Pathog* 8, e1002627.
- Schmidt, A.G., Yang, P.L., and Harrison, S.C. (2010). Peptide Inhibitors of Dengue-Virus Entry Target a Late-Stage Fusion Intermediate. *PLoS Pathog* 6, e1000851.
- Stiasny, K., and Heinz, F.X. (2006). Flavivirus membrane fusion. *Journal of General Virology* 87, 2755-2766.
- van der Schaar, H.M., Rust, M.J., Chen, C., van der Ende-Metselaar, H., Wilschut, J., Zhuang, X., and Smit, J.M. (2008). Dissecting the Cell Entry Pathway of Dengue Virus by Single-Particle Tracking in Living Cells. *PLoS Pathog* 4, e1000244.
- Wang, Q.-Y., Patel, S.J., Vangrevelinghe, E., Xu, H.Y., Rao, R., Jaber, D., Schul, W., Gu, F., Heudi, O., Ma, N.L., *et al.* (2009). A Small Molecule Dengue Virus Entry Inhibitor. *Antimicrob Agents Chemother*, AAC.01148-01108.
- WHO (2012). Dengue and severe dengue.
- Yennamalli, R., Subbarao, N., Kampmann, T., McGeary, R., Young, P., and Kobe, B. (2009). Identification of novel target sites and an inhibitor of the dengue virus E protein. *Journal of Computer-Aided Molecular Design*.
- Zhang, J., Adrian, F.J., Jahnke, W., Cowan-Jacob, S.W., Li, A.G., Iacob, R.E., Sim, T., Powers, J., Dierks, C., Sun, F., *et al.* (2010). Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature* 463, 501-506.
- Zhou, Z., Khaliq, M., Suk, J.-E., Patkar, C., Li, L., Kuhn, R.J., and Post, C.B. (2008). Antiviral Compounds Discovered by Virtual Screening of Small Molecule Libraries against Dengue Virus E Protein. *ACS Chemical Biology* 3, 765-775.
- Zompi, S., and Harris, E. (2012). Animal Models of Dengue Virus Infection. *Viruses* 4, 62-82.

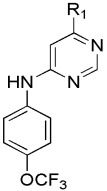

## **APPENDIX A**

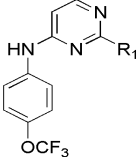
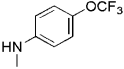


### **GNF-2 analogs identified in structure-activity relationship study**

Structures, activities, and cytotoxicities of small molecules identified as active against DENV2 in cellular assays. EC<sub>90</sub> values were determined by a six point titration curve in which molecules were pre-incubated with virus inocula; titers were determined by plaque- (DENV2) or focus-forming (DENV1, 3, 4) assay. LD<sub>90</sub> values were measured by ATP quantification with Promega CellTiterGlo kit.

							
compound ID	R <sub>1</sub>	EC <sub>90</sub> (μM)				LD <sub>90</sub> (μM)	
		DV1	DV2	DV3	DV4		
X= H	CVM-07-126-2		20 μM	20 μM	5 μM	40 μM	50 μM
	CVM-07-148-2		20 μM	15 μM	>40 μM	10 μM	80 μM
	CVM-07-148-6		7.5 μM	10 μM	>40 μM	>40 μM	100 μM
	CVM-07-127-B		17.5 μM	15 μM	30 μM	>40 μM	40 μM
	CVM-07-148-9		7.5 μM	25 μM	>40 μM	5 μM	80 μM
	CVM-07-148-7		4 μM	20 μM	30 μM	>40 μM	50 μM
	CVM-2-12-2		4 μM	5 μM	5 μM	40 μM	60 μM
	CVM-07-148-1		4 μM	17.5 μM	>40 μM	10 μM	100 μM
	CVM-07-148-8		7.5 μM	10 μM	>40 μM	20 μM	100 μM
	CVM-07-126-3		3 μM	10 μM	>40 μM	40 μM	100 μM
	CVM-07-111-1-B		20 μM	15 μM	>40 μM	40 μM	>100 μM
X= Cl	CVM-07-128-B		3 μM	15 μM	10 μM	10 μM	>100 μM

						
cmpd ID	R <sub>1</sub>	EC <sub>90</sub> (μM)				LD <sub>90</sub> (μM)
		DV1	DV2	DV3	DV4	
CVM-08-24-1		4 uM	4 uM	>40 uM	15 uM	50 uM
CVM-08-24-5		1 uM	3 uM	>40 uM	15 uM	90 uM
CVM-2-12-3		1 uM	5 uM	40 uM	17.5 uM	100 uM
CVM-07-119-2-B		1 uM	1 uM	>40 uM	10 uM	90 uM
CVM-08-24-2		7 uM	17.5 uM	>40 uM	35 uM	>100 uM
CVM-08-24-4		3 uM	7.5 uM	40 uM	7.5 uM	75 uM
CVM-08-24-3		3 uM	5 uM	>40 uM	10 uM	90 uM

						
cmpd ID	R <sub>1</sub>	EC <sub>90</sub> (μM)				LD <sub>90</sub> (μM)
		DV1	DV2	DV3	DV4	
CVM-1-100-1		20 uM	7.5 uM	>40 uM	15 uM	40 uM

						
cmpd ID	R <sub>1</sub>	EC <sub>90</sub> (μM)				LD <sub>90</sub> (μM)
		DV1	DV2	DV3	DV4	
CVM-2-12-4		1 uM	20 uM	30 uM	5 uM	>100 uM
CVM-07-120		7 uM	20 uM	>40 uM	7.5 uM	75 uM
CVM-2-21-2		3 uM	3 uM	>40 uM	30 uM	>100 uM